EVALUATION OF ANTI -TUMOR ACTIVITY OF METHANOLIC LEAF EXTRACT OF ‘‘*Cassia fistula linn.,*” AGAINST DALTON’S LYMPHOMA ASCITES - INDUCED TUMOR IN MICE

Dissertation submitted to

The Tamil Nadu Dr. M.G.R. Medical University, Chennai-32.

In partial fulfillment for the award of the degree of

MASTER OF PHARMACY

IN

PHARMACOLOGY

Submitted by

Reg.No: 261225201

Under the Guidance of

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Komarapalayam – 638183.

Tamil Nadu.

APRIL – 2014

CERTIFICATES

CERTIFICATE

This is to certify that the work embodied in this dissertation entitled ‘‘**EVALUATION OF ANTI - TUMOR ACTIVITY OF METHANOLIC LEAF EXTRACT OF ‘‘C*assia fistula linn.,”* AGAINST DALTON’S LYMPHOMA ASCITES - INDUCED TUMOR IN MICE** submitted to “The Tamil Nadu Dr. M.G.R. Medical University”, Chennai, in partial fulfillment and requirement of university rules and regulation for the award of Degree of **Master of Pharmacy** in **Pharmacology**, is a bonafide work carried out by **Mr. DILSHAD P [Reg.No.261225201]** during the academic year 2013-2014, under my guidance and direct supervision in the department of Pharmacology, J.K.K. Nattraja College of Pharmacy, Komarapalayam.

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EVALUATION CERTIFICATE

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Internal Examiner **External Examiner**

ANIMAL ETHICAL COMMITTEE CLEARANCE CERTIFICATE

We, the undersigned Chairman / members of the Animal Ethical Committee, functioning in J.K.K. Nattraja College of Pharmacy have studied the proposed research Subject / Project of **Dilshad P** entitled "**EVALUATION OF ANTI - TUMOR ACTIVITY OF METHANOLIC LEAF EXTRACT OF ''C*assia fistula linn.,"* AGAINST DALTON'S LYMPHOMA ASCITES - INDUCED TUMOR IN MICE"** applying for permission for animal usage and hereby give the Certificate of Clearance of Approval by this Ethical Committee.

Signature of the Chairman / Members of the

Animal Ethical Committee

Name of the Institution	:J.K.K.Nattraja College of Pharmacy
Station	:Komarapalayam
Date	:
Seal	:

DEPARTMENT OF BIOTECHNOLOGY
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Dr. N. Elangovan, M.Sc., Ph.D.
Assistant Professor

Monday, March 10, 2014

To,

Mr.DILSHAD
Reg No 261225201
M pharm II year
Department of Pharmacology,
J.K.K. Nattraja College of Pharmacy,
Komarapalayam

Dear Sir,

Subject: Plant authentication - Reg

.....

The plant specimen brought for identification was identified as *Cassia fistula L* Family *Fabaceae*. **Ref No:** *PU/BT/ Cassia fistula L*/Voucher specimen: 013/2014

With my best regards,

Sincerely yours,

[N. Elangovan]

DECLARATION

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I hereby declare that the dissertation entitled ‘‘**EVALUATION OF ANTI - TUMOR ACTIVITY OF METHANOLIC LEAF EXTRACT OF ‘‘C*assia fistula linn.,”* AGAINST DALTON’S LYMPHOMA ASCITES - INDUCED TUMOR IN MICE ,** has been carried out under the guidance and supervision of **Mr. S. VENKATESH M.Pharm.,** Department of Pharmacology, J.K.K. Nattraja College of Pharmacy, Komarapalayam, in partial fulfillment of the requirements for the award of degree of **Master of Pharmacy** in **Pharmacology** during the academic year 2013-2014.

I further declare that, this work is original and this dissertation has not been submitted previously for the award of any other degree, diploma associateship and fellowship or any other similar title.

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DILSA
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DEDICATED TO
ALMIGHTY,
MY BELOVED FAMILY,
TEACHERS AND FRIENDS.

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1. INTRODUCTION

1.1 TUMOR[1]:

Neoplasia literally means the process of "new growth," and a new growth is called a neoplasm. The term tumor was originally applied to the swelling caused by inflammation. Neoplasms also may induce swellings, but by long precedent, the non-euplastic usage of tumor has passed into limbo; thus, the term is now equated with neoplasm. Oncology (Greek oncos = tumor) is the study of tumors or neoplasm's. Cancer is the common term for all malignant tumors. Although the ancient origins of this term are somewhat uncertain, it probably derives from the Latin for crab, cancer-presumably because a cancer "adheres to any part that it seizes upon in an obstinate manner like the crab." Although all physicians know what they mean when they use the term neoplasm, it has been surprisingly difficult to develop an accurate definition. The eminent British Oncologist Willis has come closest: "A neoplasm is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner after cessation of the stimuli which evoked the change." We know that the persistence of tumors, even after the inciting stimulus is gone, results from heritable genetic alterations that are passed down to the progeny of the tumor cells. These genetic changes allow excessive and unregulated proliferation that becomes autonomous (independent of physiologic growth stimuli), although tumors generally remain dependent on the host for their nutrition and blood supply. As we shall discuss later, the entire population of cells within a tumor arises from a single cell that has incurred genetic change, and hence tumors are said to be clonal.

1.2 Nomenclature:

All tumors, benign and malignant, have two basic components: (1) proliferating neoplastic cells that constitute their parenchyma and (2) supportive stroma made up of connective tissue and blood vessels. Although parenchymal cells represent the proliferating "cutting edge" of neoplasm's and so determine their behavior and pathologic consequences, the growth and evolution of neoplasm's are critically dependent on their stroma. An adequate stromal blood supply is requisite, and the stromal connective tissue provides the framework for the parenchyma. In addition, there is cross-talk between tumor cells and stromal cells that

appears to directly influence the growth of tumors. In some tumors, the stromal support is scant and so the neoplasm is soft and fleshy. Sometimes the parenchymal cells stimulate the formation of an abundant collagenous stroma, referred to as desmoplasia.

1.3 DEFINITION:

Solid tumors are defined as abnormal masses of tissue that usually do not contain cysts or liquid areas. Solid tumors may be benign (not cancerous), or malignant (cancerous). A number of malignant diseases are often also categorized as "solid tumors" in the clinic such as breast cancer, cancer of the pancreas, lung, colon, etc.

Solid tumors can be split into three separate categories, depending on the type of cells from which they typically arise in the patient's body, which include:

- **Sarcomas:** Cancers arising from connective or supporting tissues such as bone or muscle.
- **Carcinomas:** Cancers arising from the body's glandular cells and epithelial cells, which line the air passages and gastrointestinal tract.
- **Lymphomas:** Cancers of the lymphoid organs such as lymph nodes, spleen, and thymus, which produce and store infection-fighting cells. Lymphoma is cancer of the lymphatic system, which is part of the immune system. The lymphatic system is a network of nodes connected by vessels which drain fluid and waste products from all the organs and structures of the body. It is also involved in producing a type of white blood cell called lymphocytes that help protect against infections. Lymphoma occurs when the lymph node cells or lymphocytes begin to multiply uncontrollably, producing malignant (cancerous) cells. Lymphoma can start almost anywhere in the body and can spread beyond the lymphatic system to other tissues and organs. Over time, lymphoma cells may replace the normal cells in the bone marrow. This can result in the inability to produce red blood cells that carry oxygen, white blood cells that fight infection, and platelets that stop bleeding.

1.4 FREE RADICALS AND ANTIOXIDANTS[2]:

Oxidative stress is a crucial etiological factor to the pathophysiology of a variety of degenerative pathological conditions such as aging, cancer, coronary heart disease, Alzheimer's disease, atherosclerosis and inflammation. Human body has multiple mechanisms especially enzymatic and non enzymatic antioxidant systems to protect the cellular molecules against reactive oxygen species (ROS) induced damage. However due to the overproduction of reactive species and/or inadequate antioxidant defense, it culminates in severe or continued oxidative stress. The harmful action of the free radical can, however, blocked by antioxidant substances, which scavenge the free radicals and detoxify the organism.

Figure1.1 Shows Free Radical Generation[3]

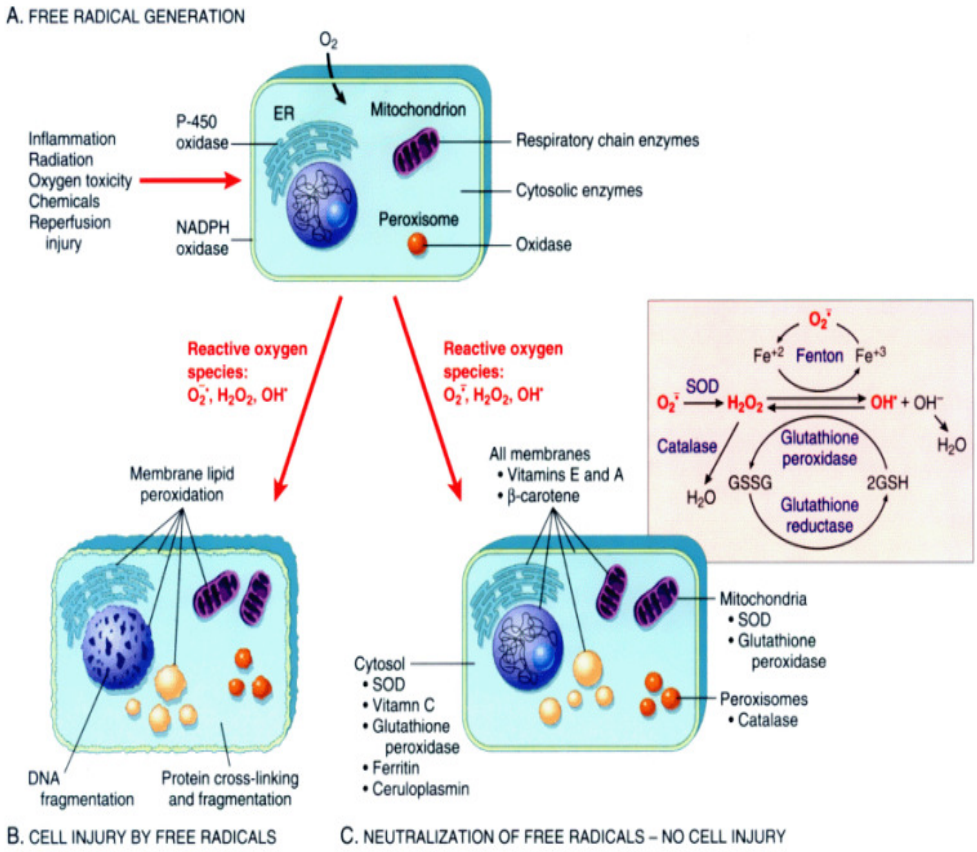
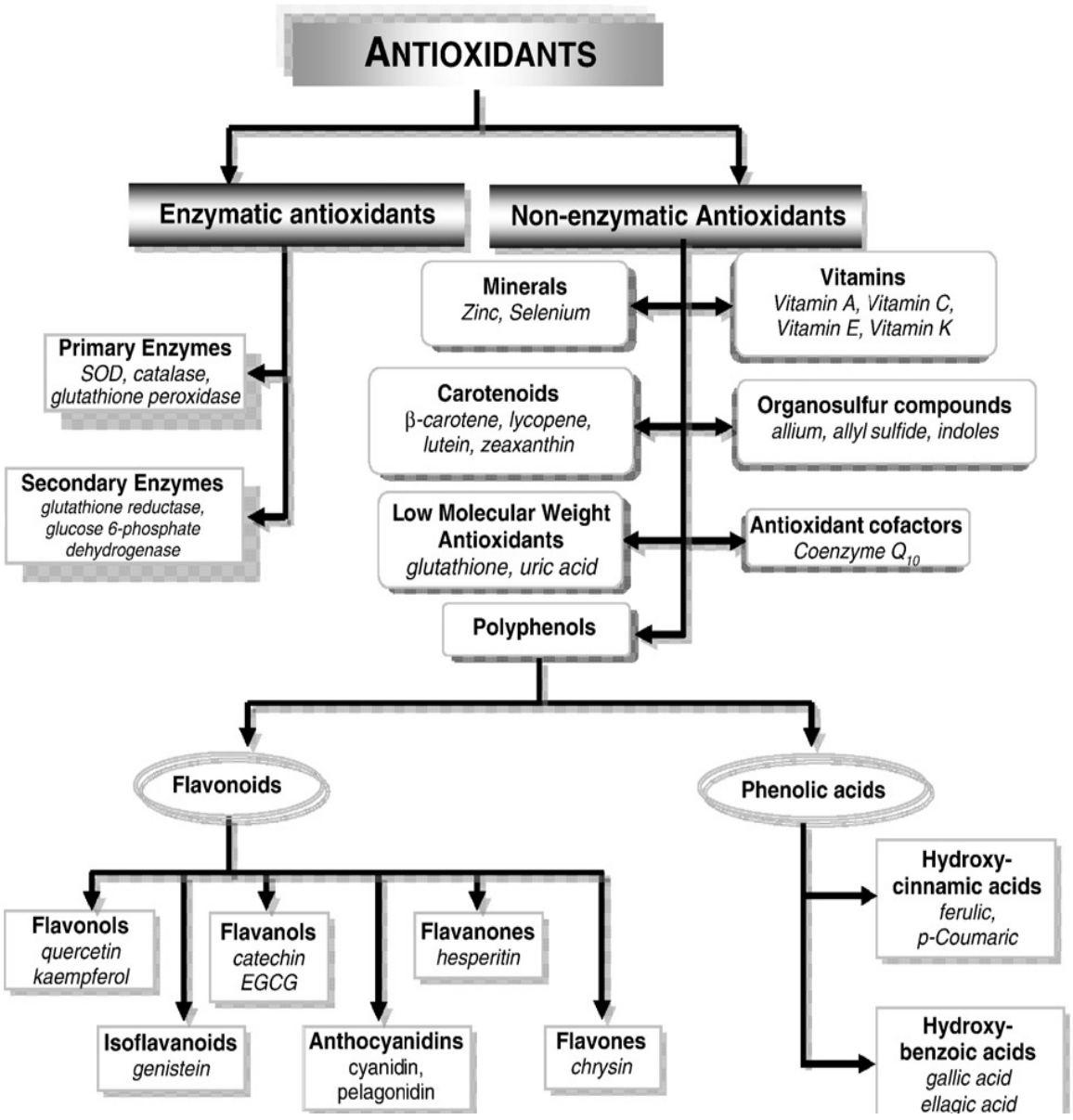


Figure1.2 Classification of Antioxidants



1.4.1 Definition and Importance of Antioxidants:

Antioxidants are substances which counteract free radicals and prevent the damage caused by them. These can greatly reduce the adverse damage due to oxidants by crumbling them. Some non-enzymatic antioxidants like uric acid, vitamin E, glutathione and CoQ10 are synthesized in the human body and they can also be derived from dietary sources. Polyphenols are the major class of antioxidants which are derived from diet. Before they react with biologic targets,preventing chain reactions or preventing the activation of oxygen to highly reactive products. Except for anaerobes, oxygen is vital for all the living systems. However, the paradox of aerobic life is that oxidative damage occurs at the key biological sites, threatening their structure and function. Oxygenic threat is met by an array of antioxidants that evolved in parallel with our oxygenic atmosphere[4]. Our body implements various antioxidants, some of which are dietary-derived antioxidants to help restrain potential free radical damage that could occur in our bodies. If one looks back into the evolution of human diet, it can be observed that in the Paleolithic age human intake of plant-derived antioxidants is considered to have been many times higher than current intake[5]. Organized agriculture has begun approximately 12,000 years ago, and stimulated a pace of dietary and social changes that deprived our biological ability to adapt to the rapid changes in environment. This has led to the hypothesis that various common diseases of civilization are rooted in a chronic divergence between our ancient nutritional programming and our contemporary dietary input[6,7]**.** A key disparity between dietary supply and physiological need may be in antioxidant nutrients. Adding to this, in modern day environment, people are exposed to a variety of toxins, which can be potent oxidants. If one combines the increasing environmental pressure of oxidant damage with our unbalanced contemporary food supply, the value of antioxidant supplementation becomes apparent. These agents should possess the characteristics like good bioavailability, stability and selectivity to the damaged or transformed cells. Formulating antioxidants is particularly interesting because of their relative lack of toxicity, preventive and therapeutic roles in diverse diseases, and encouraging evidences from epidemiology.

1.4.2 Classification of antioxidants

Antioxidants can be classified into two major groups, i.e., enzymatic and non-enzymatic antioxidants. Some of these antioxidants are endogenously produced which include enzymes, low molecular weight molecules and enzyme cofactors. Among non-enzymatic antioxidants, many are obtained from dietary sources. Dietary antioxidants can be classified into various classes**,** of which polyphenols is the largest class[8]. Polyphenols consist of phenolic acids and flavonoids. The other classes of dietary antioxidants include Vitamins, carotenoids, organo sulfural compounds and minerals (Fig. 1).

1.4.3 Role of Free Radicals and Antioxidants in Cancer:

Oxidative stress is initiated by ROS such as superoxide anion and hydrogen peroxide. Neither of these ROS is a strong oxidant, but they can be converted into more dangerous oxidants by harmful reactions in tissues[9]. Superoxide can be produced from molecular oxygen by diverse cell types via enzymatic systems including the respiratory chain, xanthenes oxidase, cyclo - oxygenase and NADPH - oxidase. It rapidly dismutates into H_2O_2, either spontaneously or enzymatically, but if superoxide collides with nitric oxide the formation of peroxy nitrite takes place[10]. H_2O_2 is formed as a product of superoxide dismutation, although some enzymes like monoamine oxidase can produce H_2O_2 directly from their substrates. Fenton or Haber-Weiss reactions catalyzed by transition metals like iron convert H_2O_2 into extremely strong hydroxyl radicals, while myeloperoxidase produces hypochlorus acid from H_2O_2. These radicals attack sensitive cellular targets like lipids, proteins and nucleic acids causing their inhibition and accelerated degradation. Thus, oxidative stress inflicts multiple levels of cellular damage, which propagates a vicious cycle. Oxidation of phospholipids and fatty acids produces reactive lipid peroxides, which in turn initiate chain reaction of lipids peroxidation in cellular membranes[11].

Some selected antioxidants and their mechanisms of action:

Antioxidant	Mechanism of action
SOD	Dismutation of superoxide to H_2O_2
CAT	Decomposes H_2O_2 to molecular oxygen and water
NAC	Scavenging of H_2O_2 and peroxide Deacetylation of precursor for GSH synthesis
GSH	Intracellular reducing agent
Lycopene	Trapping of singlet oxygen
Ellagic	Acid Scavenging of H_2O_2 Stimulation of glutathione-S-transferase
CoQ_{10}	Inhibition of lipid peroxidation Reduces mitochondrial oxidative stress
I_3C	Inhibition of DNA-carcinogen adduct formation Suppression of free radical production
Genistein	H_2O_2 scavenging
Quercetin	H_2O_2 scavenging, one of the potent antioxidant Among polyphenols
Vitamin C	Scavenging of superoxide anion by forming Semi dehydro ascorbate radical which is subsequently Reduced by GSH
Vitamin E	Direct scavenging of superoxide Upregulation of antioxidant enzymes Inhibition of lipid peroxidation

SOD=superoxide dismutase, CAT=catalase, NAC=N-acetyl cysteine, GSH= glutathione, CoQ10=coenzyme Q10, I_3C=indole-3-carbinol.

1.5 PRESENT STRATAGIES IN TREATMENT OF TUMOR:

1.5.1 Adoptive Engineered T-Cell Targeting To Activate Tumor Killing[12]:

T-cells can be used to treat some malignant diseases but many tumors avoid destruction by the immune system. The last 30 years of research has brought further understanding of T-cells and consequently the adoptive transfer of immune T-cells has shown that it may be effective against tumor in some malignancies, for example in melanoma or in lymphomas. The development of gene therapy techniques have supported the idea of 'engineered T-cells'. Attack is a pre-clinical research project focusing on the development of immune cell therapies based on the concept of genetically engineered T-cells to target cancer The strategies that produce engineered T-cells employ the transfer of tumor targeting receptors on the outside surface of the T-cells using viral vectors to help the T-cells to bypass the mechanisms of immune controls triggered by the tumor.

Aim:

Attack aims to improve engineered T-cell function and to perform pre-clinical studies which will underpin future clinical trials. With this in mind, attack will also enhance the understanding of the mechanisms involved in tumor evasion of immune control. The overall objectives are broken down within six work packages (WP):1. Optimization of two receptor - based strategies to endow the T-cells with tumor specificity (WP1 and 2). The first strategy is based in engineering T-cells to express recombinant T-cell receptors complex (TCR α and β) recognizing MHC restricted antigens at the surface of the tumor cells (WP1). The other strategy is based on chimeric immune receptors (CIR), which are scFv or small antibody molecules linked to the TCR α (WP2).

Expected results:

The project started in November 2005 and so far a website has been created for the dissemination of information within a secure portal for the members of the attack project and external pages for dissemination to the general public. The next five years will bring different sets of results established in tumor cell lines and in mouse

models as well as a variety of genetic and molecular data testing performance and specificity of the two engineered T-cell strategies.

1. Animal models will give important clues on the fundamental mechanisms of action of the engineered T-cells and the immune response they trigger. Mouse models will allow comparison of relative efficacy of T-cells redirected with scFv and TCR specific for the same antigen.
2. Animal studies will also enable the testing of different protocols aimed at improving the engineered T-cell killing function employing chemokines or cytokines that can be applied to the clinic later. State-of-the-art imaging technology, like the dorsal skin fold window chamber, will be used to follow migration of the engineered T-cells in vivo and in real time experiments.
3. The development and testing of various products from the commercial partners will define unique protocols for the selection and expansion of engineered T-cells.
4. Finally important safety data on auto-immunity, immune response against the different parts of the receptor introduced in engineered T-cells will be collected from the in vivo experiments in order to gather pre-clinical data indispensable for future clinical trials.
5. Read outs from these results will be in peer-review publications, popular articles and other published material such as poster presentation at international meetings.

1.5.2 Flavonoids:

- Flavonoids are found in higher vascular plants, particularly in the flower, leaves and bark. They are especially abundant in fruits, grains and nuts, particularly in the skins.

- Beverages consisting of plant extracts (beer, tea, wine, fruit juice) are the principle source of dietary flavonoids intake. A glass of red wine has ~200 mg of flavonoids.

- Typical flavonoids intake ranges from 50 to 800 mg/day, which is roughly 5, 50 and 100 times that of Vitamins C, and E, and carotenoids respectively.

1.5.3 Flavonoids role in cancer[13]:

Few diseases are feared more than cancer because cancerous diseases, after cardiovascular disorders and accidents, kill more people before a normal life span has been reached. Besides, the progress of cancer is often accompanied by great pain and ugly disfiguration of the body. Yet, in principle, cancer is curable if it is discovered early and treated with the best current therapeutic methods. However, radical cancer cure is fraught with considerable life-threatening dangers, loss of organs, pain, and discomfort. Besides, its treatment is expensive. Hence, it is understandable that many cancer patients look for and try milder anticancer therapies that offer some promise of a moderate,

Long - term life-saving cure. The flavonoids are some of the most promising anticancer natural products that have been tried. Related synthetic substances, e.g., flavone acetic acid, have been subjected to Phase I clinical trials already, and they may soon become adopted into the general repertoire of cytostatic treatment.

1.5.4 Flavonoids role in anti oxidants:

a. Enhance or mimic antioxidant enzymes.

b. Direct scavenging of ROS.

c. Repair damaged cellular components.

d. Pro-oxidant metal deactivation

Flavonoids and related compound are effective in scavenging DPPH radical[14], hydroxyl radical and in metal-chelating capacity. Flavonoids are found to exhibit numerous biological activities like vasodilatory, **anticarcinogenic**, antiinflammatory, antibacterial, immune-stimulating, antiallergic, and antiviral effects [15].

1.6 CELL LINES[16]:

If the specified number of cells of inoculated in to sensitive mouse strain, tumors can be developed rapidly as compared to chemical carcinogen-induced tumors and time can saved using this model.

1.6.1 METHODS INVOLVED IN CELL LINES:

1) L-1210 and P-388 cell lines are used. These cell lines are derived from mouse lymphatic leukemia and have 100% growth fraction and tumor implanted with specified number of L-1210 or P-388 cells can be predicted. The effective drug would retard the tumor growth and increase the life span of the animal. A drug, which prolongs the lifespan of the animal by 20%, is taken for subsequent studies involving testing on other transplantable tumors. Some other cell lines which can be inoculated to induce tumors are B-16(melanoma), Lewis lung carcinoma and sarcoma-180, etc. The host mouse strain for above type of cell lines is BDF, except Swiss for sarcoma-180. P-388 and L-1210 cell lines are inoculated intraperitoneally and sarcoma-180 as subcutaneously. The experiment takes about 10 days for completion.

ILS (%) =

[(Mean survival of treated group/ Mean survival of control group)-1] x100

For sarcoma-180 tumors, reduction of tumor size (tumor weight) is used to find out the inhibiting activity of solid tumors as follows.

Tumor inhibiting activity =

(average tumor weight of the treated group/average tumor weight of control group) x 100

1) Hollow-fiber technique:

Small hollow fibers (tubes 1mm in diameter and 2cm long made of plastic, polyvinylidene flouride), containing cells from human tumors are inserted underneath the skin and in the body cavity of the mouse. Each candidate drug is administered at two dosages and is tested against 12 target tumor cells in different hollow fibres. A total of about 20 compounds / week are screened by this method. Compounds that retard the growth of the cells are recommended for the next level of testing. The average length of this test is four days.

2) **Nude mouse model:**

Nude mice have been widely used to test the tumorogenicity of cells or for testing of anticancer drugs. These mice are immunologically incompetent because of absence of thymus. They neither show mitotic response in mixed lymphocyte reaction, nor generate cytotoxic effectors cell. Lack of helper T and suppressor T cells alters the antibody response of the animals to antigen. They do not show contact sensitivity and do not reject the transplanted material. They are required to be maintained under strictly sterile conditions and in a warm environment (26-280).

Some other points regarding their use are:

a. Certain tumors like melanomas and colon carcinomas grow very well in nude mice, whereas prostate carcinoma and most types of leukemia grow very poorly.
b. Large numbers of cells, usually $>10^6$ are required to be inoculated beneath the skin to get a successful tumor take.
c. Metastases are rarely observed.
d. Overall maintenance is very expensive.

1.7 INVITRO METHODS FOR SCREENING OF NEW ANTICANCER MOLECULES[16]:

Though animal models provide more predictable results, invitro testing is still preferred prior to invivo testing of a potential chemotherapeutic agent. Invitro cultures can be cultivated under a controlled environment (P^H, temperature, humidity, oxygen / carbondioxide balance etc) resulting in a homogenous batches of cells and thus minimizing experimental errors.

Advantages:

- Less time consuming
- More cost effective
- Small quantities of, and large number of compounds can be tested.
- These are easier to manage.

Disadvantages:

- Sometimes these methods may furnish false positive results (compounds show no activity invivo) or false negative results (compounds show no activity invitro but show activity invivo as they need to be biotransformedin vivo to a pharmacologically active compound).

- The role of pharmacokinetics in determining drug effects cannot be evaluated invitro.
- Geometry of solid tumors invivo is very different from that of cells growing invitro in suspension or monolayer cultures.

An ideal invitro screening method should be simple, economical, reproducible, rapid and sensitive. The assay should be applicable to large number of clinical situation as close as possible. The choice of the cell lines should be representative of clinical situation as close as possible. The range of drug concentrations used invitro should be comparable to that expected for in vivo treatment. The assay should be able to process a large number of samples quickly and in an automated fashion. Data acquisition should be simple, easily interpreted and applied. At present no such system is available. Even the most extensively studied assays will more often identify agents that will not work in an individual patient. However, the chemo sensitivity assays contribute to an active area of research and routinely used for the screening of anticancer drugs.

The goal of a screening assay to test the ability of a compound to kill cells, at the same time, the assay should be able to discriminate between replicating cells and non-replicating cells (quiescent cells that are dead or dying -Apoptosis). Different assays takes advantage of various properties of cells as mentioned below.

Table 1.1. LIST OF INVITRO SCREENING METHODS

S.No	Cell Properties	Assay
1.	Enzymatic properties	Tetrazolium salt assay(MTT)
2.	Protein content/ synthesis	Sulphorhodamine B assay
3.	DNA content/ synthesis	3H-Thmidine uptake Newer fluorescent analogues with flow cytometry
4.	Membrane integrity	Dye exclusion tests
5.	Clonogenic properties	Clonogenic assay
6.	Cell division	Cell counting assay

1.8 ROLE OF IL-2 IN TUMOROGENESIS[17]:

IL-2 is a protein of 133 amino acids (15.4 kDa). It is produced mainly by T-cells expressing the surface antigen CD4 following cell activation by mutagens or allogens under physiological conditions. IL-2 displays significant anti-tumor activity for a variety of tumor cell types since it supports the proliferation and clonal expansion of T-cells that specifically attack certain tumor types. Human IL-2 is active on mouse cells, mouse IL-2 is species-specific and is inactive on human cells. At the amino acid sequence level, there is approximately 60% similarity between mouse and human IL-2.

1.9 SIGNIFICANCE OF THE PLANTS CHOSEN FOR THE PRESENT STUDY:

The plant C***assia fistula linn*** was reported that, it has a rich amount of Flavanoids[18]. The leaves showed mainly the presence of Anthraquinone glycosides and Flavonoids. The Anthraquinone glycoside includes rhein, emodine, physion, chrysophanol (marker), Obtusin, chrysoobtusin, chryso-obtusin-2-O-β-D-glucoside, obtusifolin and chryso-obtusifolin-2-O- β -Dglucoside103 also suggest that C***assia fistula linn*** species have an anti cancer activity.

1.10 SIGNIFICANCE OF THE DALTON'S LYMPHOMA ASCITES TUMOR CELL LINES (DAL) CHOSEN FOR THE PRESENT STUDY:

1.10.1 Dalton's lymphoma Ascites tumor cell lines (DAL):

- ✓ It is a tumor cell line originally grown from a tumor of the thymus.
- ✓ It is propagated by growing as ascites tumor in mice.
- ✓ We can induce both ascites tumor and solid tumors using DAL cells.
- ✓ It is easy to maintain in vivo.

1.10.2 Maintenance of cell lines:

Tumor Cell Line and their Maintenance, Dalton's lymphoma ascites tumor cell lines (DLA), originally obtained from Amala Cancer Institute, Thrissur, Kerala were propagated as transplantable tumors in the peritoneal cavity of the mice were used for the study. The tumor cell lines were maintained by serial peritoneal cavity i.p transplantation in mice. Full-grown tumor cell-line were aspirated mouse by injecting PBS in to peritoneal cavity make cells to suspend in PBS, take that suspended solution and count the number of cells present in one ml by using tryphan blue exclusion method and adjust the cell count to 1×106 by using PBS were inject intraperitoneally in to a new healthy mouse.

1.11 OBJECTIVE OF WORK:

Cancer is one of the major diseases and challenging to medicinal system to produce potent and the site specific anti-cancer drugs. Vast number investigations are going on to find out the potent and safer anti-cancer drugs.

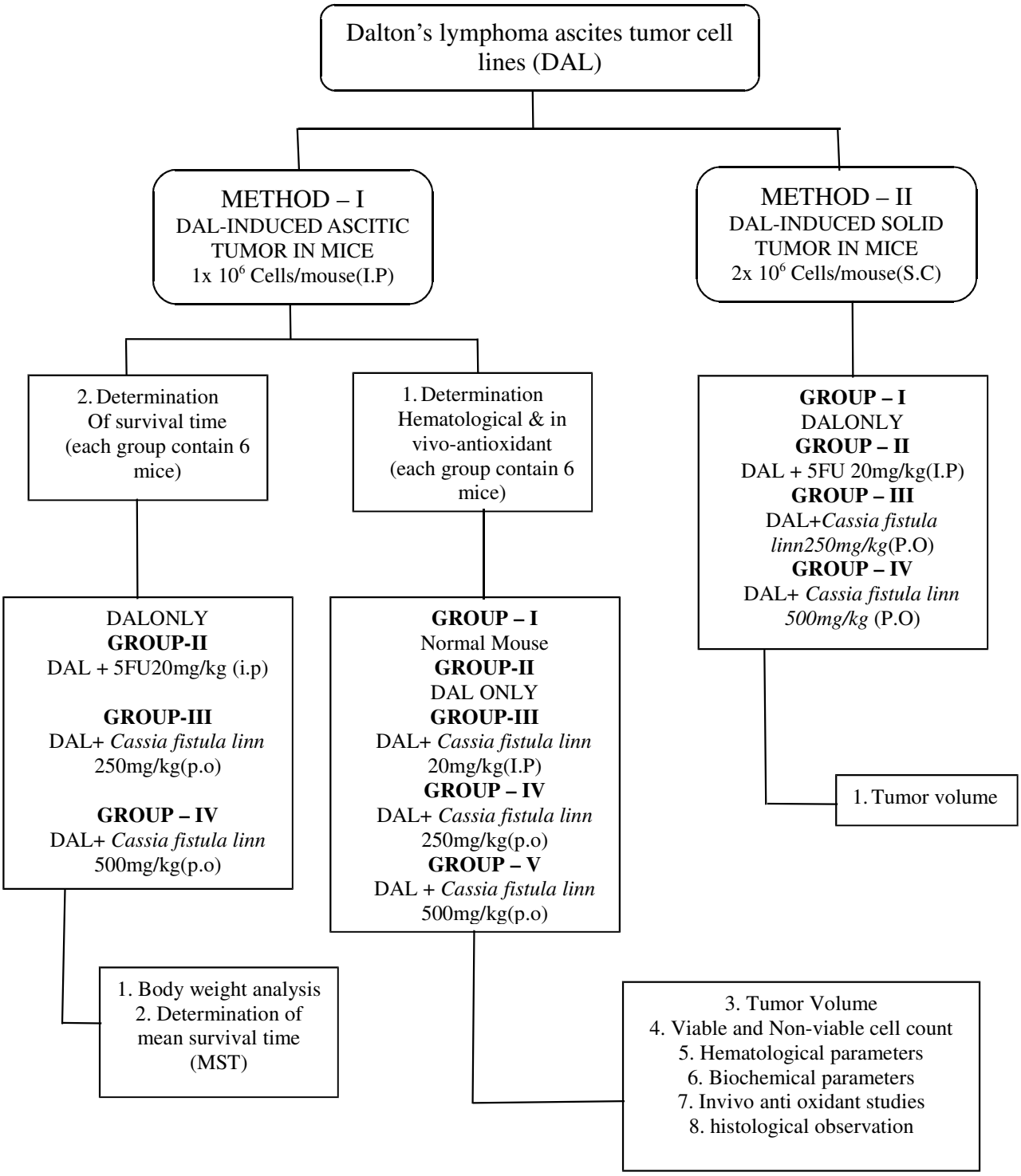
In this cancer studies, till now most of the synthetic drugs produce undesirable side effects. At the same time from plant source produce a potent and safer anti cancer drugs than compare to synthetic drugs. So in this present study, plant source was selected. C***assia fistula linn*** leaves have rich amount of Flavanoids, phenolic compounds and other active chemical constituents.

Flavanoids have a capacity to elevate the anti oxidant levels and prevent the free radical reactions. And also reported that Flavanoids have a anticancer activity a specially in breast cancer. So the present study C***assia fistula linn*** have high amount of Flavanoids content and also it proved in several hypoglycemic hypolipidimic studies.

Till now, there is no anti tumor and antioxidant study was reported on Leaves of ***Cassia fistula linn,*** for that reason this present study chooses Antitumor and Antioxidant

Status of C***assia fistula linn*** leaves against Dalton's Lymphoma Ascites-Induced Ascitic Tumor in Mice.

1.12 PLAN OF WORK



2. REVIEW OF LITERATURE

PLANT PROFILE:

Cassia fistula Linn., (Fabaceae, Caesalpinioideae), a very common plant known for its medicinal properties is a semi-wild in nature. It is distributed in various regions including Asia, South Africa, China, West Indies and Brazil [19]. It is commonly known as Amultas and in English popularly called "Indian Laburnum" has been extensively used in Ayurvedic system of medicine for various ailments. It is deciduous and mixed-monsoon forests throughout greater parts of India, ascending to 1300 m in outer Himalaya, is widely used in traditional medicinal system of India[20].

Geographical distribution: In deciduous and mixed monsoon forests throughout greater parts of India, ascending to 1300 m in outer Himalaya. In Maharashtra, it occurs as a scattered tree throughout the Deccan and Konkan[21]. The plant is cultivated as an ornamental throughout India[22].

TAXONOMIC POSITION

Kingdom: *Plantae*
Subkingdom: *Tracheobinota*
Super Division: *Spermatophyta*
Division:*Mangoliophyta*
Class: *Magnoliopsida*
Sub Class:*Rosidae*
Order:*Fabales*
Family: *Fabacae*
Genus: *Cassia*
Species: *fistula*

Vernacular Names

Bengali: *Amultash,sondal,sonali*

English: *Golden shower, Indian laburnum*

Gujarati: *Girmala*

Hindi: *Bandarlathi,bharva,suvarnaka*

Malayalam: *Tengguli,rajah*

Sanskrit: *S*araphala*,survanaka,argwadha ,rajtaru*

Tamil: *Kavani,konnai,tirukontai,sarakkonne, Raelachettu*

Telugu: *Kakkemara*

Marathi: *Bahava*

Punjabi: *Amaltaas, Kaniyaar, Girdnalee*

Oriya: *Sunaari*

Urdu: *Amaltaas*

Arab: *Khayarsambharchaiyaphruek,khuun*

Thai: *Canâfístulamansa,chácara ,Guayaba*

Spanish: *Bâtoncasse, cassedoux, casseespagnol cimarrona*

Trade name: *Indian laburnum*

Morphology

It is a deciduous tree with greenish grey bark, compound leaves, leaf lets are each 5-12 cm long pairs. A semi - wild tree known for its beautiful bunches of yellow flowers and also used in traditional medicine for several indications [23]. A fruit is cylindrical pod and seeds many in black, sweet pulp separated by transverse partitions. The long pods which are green, when unripe, turn black on ripening after flowers shed [24]. Pulp is dark brown in colour, sticky, sweet and mucilaginous, odour characteristic, and somewhat disagreeable [25]. Drug occurs in flat or curved thick pieces; outer surface smooth to rough with warty patches; greenish grey to red; inner surface rough, reddish with parallel striations; fracture, laminate; odour, sweet and characteristic; taste, astringent [26]. A tree 6-9 m high; trunk straight; bark smooth and pale grey when young, rough and dark brown

when old; branches spreading, slender. Leaves 23-40 cm long; main rhachis pubescent; stipules minute, linear -oblong, obtuse, pubescent. Leaflets 4-8 pairs, ovate or ovate-oblong, acute, 5-12.5 by 3.8- 9.5cm, bright green and glaborous above, paler and silvery pubescent beneath when young, the midrib densely pubescent on the underside, base cuneate; main nerves numerous, close, conspicuous beneath; petiolules 6-10 mm long, pubescent or glaborous. Flowers in lax racemes 30-50 cm. long; pedicels 3.8- 5.7 cm. long, slender, pubescent and glaborous. Calyx 1 cm long divided to the base, pubescent; segments oblong, obtuse. Corolla 3.8 cm across, yellow; stamens all antheriferous. The pods are pendulous, cylindric, nearly straight, smooth, shining, brown-black, indehiscent, with numerous (40-100) horizontal seeds immersed in a dark coloured sweetish pulp. Seeds broadly ovate, 8mm. long, slightly less in breadth, and 5mm thick [27]. The fruit pods are 40-70 cm long and 20-27mm in diameter, straight or slightly curved, smooth but finely striated transversely, the striations appearing as fine fissures. The rounded distal ends bear a small point marking the position of the style. The dorsal suture appears as a single vascular strand and the ventral suture as two closely applied strands. Internally the pod is divided by thin, buff coloured, transverse dissepiments at intervals of about 0.5cm. Each compartment contains one seed which is flat, oval, reddish brown with a well marked raphe. The seed contains a whitish endosperm in which the yellowish embryo is embedded [28].

Traditional Medicinal Uses

The root is prescribed as a tonic, astringent, febrifuge and strong purgative [29-33]. Extract of the root bark with alcohol can be used for backwart fever. The roots are used in chest pain, joint pain, and migraine and blood dysentery. The extract of the root lowered the blood sugar level up to 30 percent[34]. Root is useful in fever, heart diseases, retained excretions and biliousness[35]. The aqueous extract of the root bark exhibits anti-inflammatory activity. The root is used in cardiac disorders biliousness, rheumatic condition, hemorrhages, wounds, ulcers and boils and various skin diseases[36]. *Cassia fistula Linn leaves* are crushed to prepare a thick paste and mixed with coconut oil. This paste is applied over the burnt skin twice a day[37, 38]. The stem bark is used against amenorrhoea, chest pain and swellings. The bark possess tonic and antidysentric

properties, it is also used for skin complaints, the powder or decoction of the bark is administered in leprosy, jaundice, syphilis and heart diseases[39].

Cassia fistula Linn

The leaves extract reduced mutagenecity in *E. coli* [40]. The leaves are laxative and used externally as emollient, a poultice is used for chilblains, in insect bites, swelling, rheumatism and facial paralysis[41-43]. Leaves posses anti periodic and laxative properties, the leaves are used in jaundice, piles, rheumatism ulcers and also externally skin eruptions, ring worms, eczema. The leaves and bark mixed with oil are applied to pustules, insect bites[44]. Juice of leaves is used in skin diseases[45, 46]. Juice of leaves is useful as dressing for ringworm, relieving irritation and relief of dropsical swelling. The pulp of the fruit around the seeds is a mild purgative [47]. Leaves and flowers are both purgative like the pulp[48]. Ashes from burnt pods mixed with little salt are used with honey taking 3- 4 times to relieve cough. Fruits are used as catharatic and in snake bite[49]. Flowers and pods are used as purgative, febrifugal, biliousness and astringent. The ethanolic 50% extract of pods show antifertility activity in female albino rats. The heated pods are applied to swellings on the neck due to cold. The fruits are reported to be used for asthma[50]. Pulp is given in disorders of liver. The drug is used as analgesic as an antipyretic, it is a remedy for malaria and fever. It is also applied in blood poisoning, anthrax and antidysentric, leprosy and antidiabetic, for the removal of abdominal obstruction[51]. The extract of the flower inhibits the ovarian function and stimulate the uterine function in albino rats. Fruits are used in the treatment of diabetes, antipyretic, abortifacient, demulcent lessens inflammation and heat of the body; useful in chest complaints, throat troubles, liver complaints, diseases of eye and gripping[52]. The fruit pulp is used for constipation, colic, chlorosis and urinary disorders[53]. The seeds are emetic, used in constipation and have cathartic properties. The seeds are slightly sweet and possess laxative, carminative, cooling, improve the appetite and antipyretic activity[54]. They are useful in jaundice, biliousness, skin disease and in swollen throat[55]. A seed dried produce marked hypoglycaemic activity. Seed powder is used in amoebiasis.

Phytochemical Profile

A majority of the ascribed biological effects of *C. fistula* extracts have been attributed to their primary and secondary metabolite composition. Primary metabolite analysis has essentially been focussed on the seed, pollen, fruit, leaf and pod. The seeds are rich in glycerides with linoleic, oleic, stearic and palmitic acids as major fatty acids

together with traces of caprylic and myristic acids. It has been reported that the stem bark of *C. fistula* is also a potential source of lupeol, ß-sitosterol and hexacosanol. In an earlier study it was reported that one of the major carbohydrates in the seeds was galactomannan consisting of 8 different types of sugar moieties. A detailed biochemical analysis of the flower's pollen, suspected to play a significant allergenic role, showed a protein composition of 12% with appreciable amounts of free amino acids such as phenylalanine, methionine, glutamic acid and proline. Carbohydrate, lipid and free amino acid contents were of the order of 11.75, 12 and 1.42%, respectively[56]. The edible fruit tissue of the Indian laburnum fruit was reported to be a rich source of potassium, calcium, iron and manganese than fruits like apple, apricot, peach, pear and orange The protein (19.94%) and carbohydrate (26.30%) contents are indicative of the potential of the fruit to be an important source of nutrients and energy. Apolar compounds including 5-nonatetracontanone, 2- hentriacontanone, triacontane, 16- hentriacontanol and sitosterol along with an oil (probably an isoprenoid compound) showing antibacterial activity have also been isolated in *C. fistula* pods[57]. *C. fistula* plant organs are known to be an important source of secondary metabolites, notably phenolic compounds. Fistucacidin, an optically inactive leucoanthocyanidin (3, 4, 7,8,4'pentahydroxyflavan) was first extracted from the heartwood. The presence of kaempferol and a proanthocyanidin whose structure has been established as a leucopelargonidin tetramer having a free glycol in the acetone extract of the flower has been documented. Proanthocyanidins containing flavan-3-ol (epiafzelechin and epicatechin) units with an abnormal 2S-configuration have also been observed in pods together with the common flavan-3-ols and proanthocyanidins like catechin, epicatechin, procyanidin B-2 and epiafzelechin[58].

LITERATURE REVIEW:

Siddhuraju P.,et al studied that , the investigation suggest that the antioxidant properties of 90% ethanol extracts of leaves, and 90% methanol extracts of stem bark, pulp and flowers from *Cassia fistula*. The antioxidant activity power was in the decreasing order of stem bark, leaves, flowers and pulp and was well correlated with the total polyphenolic content of the extracts. The reason for low antioxidant activity in the flower and pulp fractions could be the presence of some pro oxidants, such as chrysophanol and reducing sugars which dominate the antioxidant compounds present in the extracts. Thus, the stem bark had more antioxidant activity in terms of reducing power, inhibition of peroxidation, O2 and DPPH radical scavenging ability [59]. It has been reported that aqueous extract of *Cassia fistula Linn*. flowers (ACF) was screened for its antioxidant effect in alloxan induced diabetic rats. An appreciable decrease in peroxidation products vizthiobarbituric acid reactive substances, conjugated dienes, hydroperoxides was observed in heart tissues of ACF treated diabetic rats. The decreased activities of key antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione in diabetic rats were brought back to near normal range upon ACF treatment. These results suggest that ACF has got promising antioxidative activity in alloxan diabetic rats.

Barthakur N.,et al studied that, the methanol extract of seeds of *C. fistula* was tested for different pharmacological actions in mice. The extract significantly potentiated the sedative actions of sodium pentobarbitone, diazepam, meprobamate and chlorpromazine. It also potentiated analgesia induced by morphine and pethidine in a dose-dependent manner. The extract also influenced behaviour in mice[60].

Kumar A.,et al studied that, the potential of *Cassia fistula* to treat the infected wound on albino rat model was investigated[61]. The alcohol extract of *C. fistula* leaves was analyzed for antibacterial effect against *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853. Formulated ointment was topically applied on the infected wound. Wound reduction rate, histological analysis, biochemical analysis, and gelatin zymography were obtained to assess the healing pattern. *C. fistula* treated rats showed,

better wound closure, improved tissue regeneration at the wound site, and supporting histopathological parameters pertaining to wound healing. Biochemical analysis and matrix metalloproteinases expression correlated well with the results thus confirming efficacy of *C. fistula* in the treatment of the infected wound. Along with the other activities such as antitumor, antioxidant, hypoglycemic, hepatoprotective, antibacterial, hypocholesterolaemic, and antidiabetic activity, the healing potential of *C. fistula* provides a scientific rationale for the traditional use of this plant in the management of infected dermal wound and can be further investigated as a substitute to treat infected wounds without using synthetic antibiotics.

Hegde Chaitra R.,studied that , the hexane, chloroform, ethyl acetate, methanol and water extracts from the flower of *Cassia fistula* were tested against bacteria and fungi. All the extracts exhibited antibacterial activity against Gram positive organisms with minimum inhibitory concentrations (MIC) between 0.078 and 2.5 mg/ml. Among the Gram negative bacteria, only *Pseudomonas aeruginosa* was susceptible to the extracts. Ethyl acetate crude extract was fractionated using chromatographic techniques. A crystal was isolated, which was confirmed as 4-hydroxy benzoic acid hydrate using X-ray crystallography. It exhibited antifungal activity against *Trichophytonmentagrophytes* (MIC 0.5 mg/ml) and *Epidermophytonfloccosum* (MIC 0.5 mg/ml). Three lectins, i.e. CSL-1, CSL-2 and CSL-3, purified from the *Cassia fistula* seeds, were tested for their antibacterial activities against different pathogenic bacteria, i.e. *Bacillus subtilis, B. megaterium, Streptococcus haemolyticus, Streptococcus aureus, Sarcinalutea, Shigellasonnei, scherichia coli, Klebsiellasp.,Shigellashiga, Shigellaboydii, Shigellaflexneri, Shigelladysenteriae, Salmonella typhi*and*Pseudomonas aeruginosa*, using 30 micro g/disc. CSL-3 was active against all bacterial strains and showed strong activity against *B. megaterium*, *Streptococcus haemolyticus* and *Shigellaboydii*. CSL-2 showed poor activity against most of the bacterial strains and has strong activity against only *Streptococcus haemolyticus*. CSL-1 was inactive against all the bacterial strains except *Streptococcus haemolyticus* and *Sarcinalutea*. All the lectin significantly affected the mortality rate of brine shrimp. Among them, CSL-2 was highly toxic (6.68 micro g/ml) followed by CSL-1 (10.47 micro g/ml) and CSL-3 (13.33 micro g/ml). Aqueous

extract of *C. fistula* in disc diffusion method showed significant activity against *S. aureus* but not against other bacteria tested. Alcoholic extract showed greater inhibition against *S.aureus* compared to aqueous extract. One of the field isolates of *S. aureus* resistant to chloramphenicol was also susceptible to the alcoholic extract of *C. fistula*. Zones of inhibition of alcoholic and aqueous extracts were in the range of 7.0-12.0 mm and 7.0-11.6 mm, respectively. MIC values of the alcoholic extracts against *S. aureus* were in the range of 0.78-6.25 mg/ml [62, 63].

Sen A.B., et al studied that , the effects of methanolic extract (ME) of *Cassia fistula* seed on the growth of Ehrlich ascites carcinoma (EAC) and on the life span of tumor bearing mice were studied. ME treatment showed an increase of life span, and a decrease in the tumor volume and viable tumor cell count in the EAC tumor hosts. Cytological studies have revealed a reduction in the mitotic activity, and the appearance of membrane blebbing and intra cytoplasmic vacuoles in the treated tumor cells. Improvement in the haematological parameters following ME treatment, like haemoglobin content, red blood cell count and bone marrow cell count of the tumor bearing mice have also been observed. The results of the present study suggest that ME of *C. fistula* seed has an antitumor activity. Haematological studies have revealed that out of the three doses of ME, ME at the dose of 100 mg/kg has shown better results than at the doses of 200 and 300 mg/kg. The exact mechanism by which ME mediates its antitumor effect is still to beelucidated. Cytological changes indicate that ME might be having a direct tumorocidal effect on the tumor cells [64, 65].

Gupta M., et al studied that , the petroleum ether extract of seeds of *Cassia fistula* was screened for the antifertility activity in proven fertile female albino rats at the doses 100, 200 and 500 mg/kg b.wt./day. Oral administration of the extract to mated female rats on days 1-5 of pregnancy resulted in a decline in the fertility index, numbers of uterine implants and live foetuses in a dose dependent manner as was confirmed by laparotomy on day 15 of pregnancy. The extract (100 mg/kg b.wt.) exhibited weak estrogenic activity when given alone and tested in immature bilaterally ovariectomized female albino rats, but exhibited slight antiestrogenic activity when administration along with estradiol valerate (0.1 mg/kg b.wt.). Blood sugar and haematological parameters were within

normal range. Thus, the results of the present study indicate that the petroleum ether extract of *Cassia fistula* seeds possesses pregnancy terminating effect by virtue of anti-implantation activity[66].

Abu Sayeed M., et al studied that, the effectiveness of *Cassia fistula* in the treatment of leishmaniasis, the efficacy of concentrated boiled extract and hydroalcoholic extract of *C. fistula* on leishmaniasis was compared with intralesional injection of Glucantime [meglumineantimonate] in this study. 63.6% of patients treated with the concentrated boiled extract, 52.7% of patients treated with the hydroalcoholic extract, and 45.5% of patients treated with Glucantime. In total, 22 patients (40%) given the concentrated boiled extract of *C. fistula*, 20 patients (36.4%) given the hydroalcoholic extract of *C. fistula*, and 36 patients (65.5%) of the Glucantime group showed complete cure. The efficacy in the third group was significantly higher than the first ($P<0.02$) and second groups ($P<0.005$), but there was no difference between the efficacy of concentrated boiled extract and hydroalcoholic extract of *C. fistula*. These results show that this plant could be used topically along with Glucantime for decreasing the time and dose of treatment with Glucantime. The potential of *Cassia fistula* boiled extract in the treatment of cutaneous leishmaniasis, to evaluate the efficacy of intra lesional meglumine antimonate –

C. fistula fruit gel combination for the treatment of cutaneous leishmaniasis. A total of 140 patients with cutaneous, one group received intra lesional meglumine antimonate injection and *C. fistula* fruit gel, and the second group (control) was treated with intra lesional meglumine antimonite plus placebo gel. Improvement was defined as complete cure, partial cure and treatment failure. At week 12, forty-seven (67.1%) patients in the experimental group achieved complete cure, compared to 29 (41.4%) patients in the control group ($P<0.001$). Results indicate that the *C. fistula* fruit gel increases the efficacy of intra lesional meglumine antimonate for the treatment of cutaneous leishmaniasis. Combination therapy with intra lesional meglumine antimonate and *C.fistula* fruit gel should be considered for the treatment of acute cutaneous leishmaniasis[67].

3. MATERIALS AND METHODS

3.1 Collection and authentication:

The Leaves of *Cassia fistula linn* were collected locally and authenticated by Dr.N.Elangovan, M.Sc., Ph.D. Assistant professor, Department of Biotechnology, Periyar University, Salem , Tamilnadu, India.

3.2 Extraction Procedure:

3.2. A. Extraction of steam and bark of *Cassia fistula Linn.,*

The Leaves of *Cassia fistula linn* were locally collected in month of November. The Leaves were chopped, air dried for a week and powered. The powder was then extracted in petroleum ether to remove fatty material using soxhlet extractor. Then the material was air dried and again subjected to extraction with methanol by using soxhlet continuous extraction until the colour of the material disappears. The obtained extract was concentrated to remove excess of remaining methanol.

3.3. Preliminary Phytochemical analysis of Methanolic extract of Leaves of *Cassia fistula Linn.*

3.3.1 Chemical tests:

A) Test for carbohydrates:

1) Molisch Test:

In this a small amount of leaves of cassia fistula linn extract is treated with a-naphthol and concentrated sulphuric acid along the sides of the test tube. Purple colour or reddish violet colour at the junction between two liquids was formed. It indicates presence of carbohydrates.

2) Fehling's Test:

In this small amount of test extract is treated with Equal quantity of Fehling's solution A and B is and Heat gently, brick red precipitate was formed. It indicates presence of carbohydrates.

3) Benedict's test:

To the 5 ml of Benedict's reagent, added 8 drops of extraction solution. Mixed well, boiling the mixture vigorously for two minutes and then cool. Red precipitate was formed. It indicates presence of carbohydrates.

4) Barfoed's test:

To the 5 ml of the Barfoed's solution added 0.5 ml of extraction solution and mixed well and heated to boiling, red precipitate was formed. It indicates presence of carbohydrates.

B) Test for Alkaloids:

About one gram of the powdered sample was extracted with 10 mL of 10% hydrochloric acid by boiling for five minutes on a water bath. The extract was filtered and the pH of the filtrate was adjusted to about 6 by adding a few drops of dilute ammonia solution and tested with litmus paper after which few drops of Dragendorff's, Mayer's and Wagner's reagent were added separately to aliquots of the filtrate in the test tubes. A reddish brown, cream and reddish brown precipitate respectively indicates a positive test.

C) Test for Steroids and Sterols:

1) Libermann Burchard test:

Leaves of *Cassia fistula linn* is dissolved in 2 ml of chloroform in a dry test tube. Then added10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid. The solution becomes red, then later it was not changed to blue and bluish green colour it indicates absence of steroids and sterols.

2) Salkowski test:

Leaves of *Cassia fistula linn* was dissolved in chloroform and adds equal volume of conc. sulphuric acid. Bluish red cherry red and purple color is not formed in chloroform layer, and also green fluorescence was not formed indicate absence of steroids and sterols.

D) Test for Glycosides

1) Legal's test:

The extract Sample is dissolved in pyridine sodium nitropruside solution is added to it and made alkaline. Pink red colour is produced. Indicates presence of glycosides.

2) Baljet test:

To the extract sample, sodium picrate solution is added. Yellow to orange colour is produced. Indicates presence of glycosides.

3) Borntrager test:

Added a few ml of dilute sulphuric acid to the test solution. Boiled, filtered and extract the filtrate with ether or chloroform. Then organic layer is separated to which ammonia is added, pink, red or violet colour is produced in organic layer. Indicate presence of glycosides.

4) Keller Killiani test:

Sample is dissolved in acetic acid containing trace of ferric chloride and transferred to the surface of concentrated sulphuric acid. At the junction of liquid reddish brown color is produced which gradually becomes blue. Indicate presence of glycosides.

E) Test for Saponins:

About one gram of the powdered sample was boiled with 10 ml of distilled water for ten minutes. The samples were filtered while hot, cooled and the following tests were carried out.

1) Frothing:

2.5 mL of the filtrate was diluted to 10mLs with water and was shaken vigorously for 2 minutes. Frothing observed indicates a positive test.

2) Emulsification:

2.5mL of the filtrate was shaken vigorously for 2 minutes with a few drops of olive oil. An emulsified layer indicates a positive test.

F) Test for Flavanoids:

A small quantity of the extract was dissolved in dilute sodium hydroxide and hydrochloric acid was added to the mixture. A yellow solution that turns colourless on addition of hydrochloric acid indicates the presence of flavonoids.

G) Test for Triterpenoid:

In the test tube, 2 or 3 granules of tin was added, and dissolved in a 2ml of thionyl chloride solution and test solution was added. Pink colour is produced which indicates the presence of Triterpenoid.

H) Test for Protein and Amino acid:

1) Biuret test:

Added 1 ml of 40% sodium hydroxide and 2 drops of 1% copper sulphate to the extracts, a violet colour formed indicates the presence of proteins.

2)Ninhydrin test:

Added 2 drops of freshly prepared 0.2% ninhydrin reagent to the extracts and heated. A blue colour developed indicating the presence of proteins, peptides or amino acids.

3) Xanthoprotein test:

To the extracts, added 20% of sodium hydroxide. Orange colour was formed indicates presence of aromatic amino acid.

3.4. DETERMINATION OF ACUTE ORAL TOXICITY (LD_{50}) OF LEAVES OF *Cassia fistula linn* [68]

Table 3.1.Test substance details

Name of the test substance	Methanolic extract of **Leaves of *Cassia fistula Linn.***
Colour of the test substance	Brownish green
Nature of the test substance	Sticky

Table 3.2. Experimental protocol

Name of the study	Acute toxicity
Guideline followed	OECD 423 method-acute toxic class method
Animals	Healthy young adult Swiss albino mice, nulliparous, non-pregnant
Body weight	25-30 g
Sex	Female
Administration of dose and volume	2000 mg/kg body weight, single dose in 0.5 ml
Number of groups and animals	3 groups and 9 animals
Route of administration	Oral by using mice oral feeding needle
Vehicle	Distilled water

Table 3.3.Housing and feeding conditions

Room temperature	22°C ± 3°C
Humidity	40-60%
Light	12 h : 12h (light : dark cycle)
Feed	Standard laboratory animal food pellets with water *ad libitum*

Table 3.4. Study period and observation parameters

Initial once observation	First 30 minutes and periodically 24 h
Special attention	First 1-4 h after drug administration
Long term observation	Upto 14 days
Direct observation parameters	Tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma.
Additional observation parameters	Skin and fur, eyes and mucous membrane, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern etc.

The time of death, if any, is recorded. (Complete observations: annexure I). After administration of the Leaves of ***Cassia fistula Linn***, food is withheld for a further 1-2 hours.

Study procedure:

Acute oral toxicity was performed as per organization for economic co-operation for development (OECD) guideline 423 method. The **Leaves of *Cassia fistula linn*** substance was administered in a single dose by gavage using specially designed mice oral needle. Animals are fasted 3 h prior to dosing (food was withheld for 3 h but not water). Following the period of fasting animals was weighed and test substance was administered orally at a dose of 500, 1000 and 2000 mg/kg. After the **Leaves of *Cassia fistula linn*** substance administration, food was withheld 2 hrs in mice. Animals were observed individually after atleast once during the first 30 minutes, periodically during the first 24 hrs, with special attention given during the first 4 hrs, and daily thereafter, for a total of 14 days.

3.5. ESTIMATION OF *INVITRO* ANTIOXIDANT ACTIVITY METHANOLIC EXTRACTS OF LEAVES OF *Cassia fistula linn.*

3.5.1 DPPH photometric assay:[69]

Reagents:

- Diphenyl-2-picrylhydrazyl (DPPH)
- Ethanol

Principle:

DPPH is purple in colour (λ max 517 nm) and in the presence of a substance (A-H), capable of donating an electron or a hydrogen atom, it loses its radical character and becomes colourless.

Procedure:

The free radical scavenging activity of the extract was measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH radical method (Blois, 1958). Sample stock solutions (1.0 mg/ml) were diluted to final concentrations of 50, 40, 30, 20, 10 µg/ml, in ethanol. One ml of a 0.3 mM DPPH ethanolic solution was added to 2.5 ml of sample solutions of different concentrations, and allowed to react at room temperature. After 30 minutes the absorbance values were measured at 518 nm and converted into percentage of antioxidant activity (AA) using the following formula:

$$AA\ \% = 100 - \{ [(ABS_{SAMPLE} - ABS_{BLANK}) \times 100] / ABS_{CONTROL}\}$$

Ethanol (1.0 ml) plus compound solution (2.5 ml) was used for blank. DPPH solution (1.0 ml; 0.3 mm) plus ethanol (2.5 ml) was used for negative control. The positive controls were those using the standard solutions. Average percent of antioxidant activity from three separate tests were calculated.

3.5.2 $ABTS^+$ RADICAL SCAVENGING ASSAY:

Scavenging of 2,2-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) Diammonium salt (ABTS) Radical cation.[70].

Reagents:

- 4.9 mM ammonium persulfate solution
- 14 mM ABTS

Procedure:

$ABTS^+$ radical was freshly prepared by adding 5 ml of 4.9 mM ammonium persulfate solution to 5 ml of 14 mM ABTS solution and kept for 16 h in dark. This solution was diluted with ethanol (99.5%) to yield an absorbance of 0.70±0.02 at 734 nm and the same was used for the assay. To 950 µl of ABTS radical solution, added 50 µl of extract solutions (25-500 µg/ml) and the reaction mixture was vortexed for 10 sec. After 6 minutes the absorbance was recorded at 734 nm and compared with the control ABTS solution. Percentage inhibition was calculated from the formula[38].

Percentage inhibition= [1−(absorbance of test/absorbance of control)]×100

3.5.3 DCF/AAPH assay (TRAP)[71]:

Reagents:

1. Dichlorofluorescin-diacetate(DCF)
2. 0.01N NaOH
3. Sodium phosphate buffer (25 mM, pH 7.2)

Procedure:

An azo initiator, AAPH, was used to produce peroxyl radicals, and the scavenging activity of the LEAVES OF *CASSIA FISTULA LINN* extracts was monitored via the spectrophotometric analysis of 2,7- dichlorofluorescin-diacetate. The activation of DCF was achieved by mixing DCF (3.41 µl of 50µg/ml solution) and NaOH (1.75 ml of 0.01N solution) and allowing the mixture to stand for 20 min before adding 18.25 ml of sodium phosphate buffer (25 mM, pH 7.2). The reaction mixture contained 10µl of extract (diluted to final concentrations of 500, 250, 100, 50 and 25 µg/ml), 170µl activated DCF solution and 20 µl of 600 mM AAPH (adjusted to a final concentration of 60 mM). The reaction was initiated by adding the AAPH solution. After 10 min, the absorbance was read at 490 nm using a Specrophotometer.

3.5.4 FERRIC REDUCING ABILITY OF PLASMA (FRAP) EXPRESSED AS A FUNCTION OF TIME[72]

Reagents:

1. Acetate buffer (500 mM/l)
2. Tripyridyltriazine (TPTZ) (10 mM/l)
3. Ferric chloride (20 mM/l)

Procedure:

FRAP agent was prepared by mixing 25 ml of acetate buffer (500 mM/l) with 2.5 ml of tripyridyltriazine (TPTZ) (10 mM/l) and 2.5ml of ferric chloride (20 mM/l) solution. The reaction mixture contained 300 µl of freshly prepared FRAP reagent warmed to 37º C, added to 10 µl of test along with 30 µl of water. Absorbance of this solution was taken at 593 nm, just after 4 min from the time of addition of FRAP reagent. An increase in absorbance indicated enhanced reducing potential of plasma. Quantitative calculation for each sample was done using an equation obtained from the standard curve of Fe^{++}-TPTZ.

The equation used: Absorbance = 0.274 x µM of Fe^{++} + 0.114 [R^2 = 0.974]

3.6 SCREENING OF IN-VITRO CYTOTOXICITYOF METHANOLIC EXTRACTS OF LEAVES OF *Cassia fistula linn*[73]

3.6.1 Tryphan Blue Dye Exclusion Method

Requirements:

a) Daltons lymphoma ascites (DAL) cells
b) Phosphate buffer saline solutions
c) Haemocytometer
d) Tryphan blue solution (0.4%)

Procedure:

Dalton lymphoma ascites (DAL) cells were aspirated from the peritoneal cavity of mice and washed three times in phosphate buffer saline. Take 0.2ml of cell suspension (DAL), 0.3ml of PBS and 0.5ml of 0.4% Tryphan blue were mixed and kept aside for 5min and not more than 15min. From this one drop of solution was taken on a neubauer chamber and a cover slip is placed. This is placed on Haemocytometer and the viable and non-viable cells were counted under 10X power. Viable cells don't take colour

and these cells appear in white colour on blue background. Non-viabe cells (dead cells) take blue colour and give dark blue shading to the cells and cell count was calculated using formula

Cell count = No. of cells x Dilution factor × volume factor

One million cells were incubated with different concentrations of the LEAVES OF *CASSIA FISTULA* LINN extract (10, 25, 50, 100 and 200 µg/ml) and the volume was made up to 1.0 ml using PBS & Control tubes contained only cell suspensions. The assay mixture was incubated for 3 hr at 37° C .The viability of the cells was then determined using Tryphan blue dye exclusion method percentage Cytotoxicity a was calculated using formula.

% Cytotoxicity = (Total cells-Dead cells) ×100/Total cells

3.6.2 TETRAZOLIUM SALT ASSAY (MICROCULTURE TETRAZOLIUM TEST OR MTT)[74]:

Principle:

MTT assay is an internationally accepted invitro method for anticancer drug screening. MTT assay utilizes a color reaction as a measure of viable cells. The assay is dependent on the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazolium salt to a blue formazan product by the mitochondrial dehydrogenase of viable cells/metabolically active cells. The intensity of blue colored formazan produced is directly proportional to the cell viability.

Procedure:

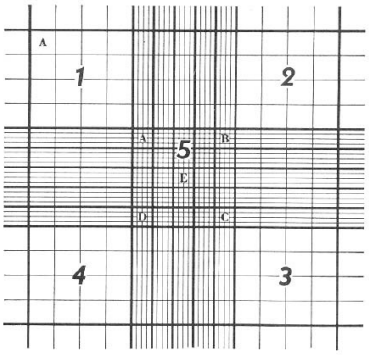
The cells from a particular cell line(HeLa) when in log phase of growth are trypsinized, counted in a hemocytometer and adjusted to appropriate density in a suitable medium and then inoculated in different multiwall plates(usually 96-well plates). The cells are treated with various concentrations of LEAVES OF CASSIA FISTULA LINNfor 1day after which MTT dye is added in each well and plates are incubated at 370 for 4h in a CO_2 incubator. The plates are then taken out of incubator and dark blue colored formazan crystals are thoroughly dissolved in Isopropanol of DMSO at room

temperature. The plates are then read on an ELISA reader at 570nm. The percent cell viability with respect to control is calculated using the formula.

% cell viability = (optical density of treated cells/optical density of control cells) ×100

3.7. EVALUATION OF IN VIVO ANTICANCER ACTIVITY OF METHANOLIC EXTRACTS OF LEAVES OF *Cassia fistula linn*[75].

Animal used:



Inbred female Swiss mice of 2 months age, weighing 20 ± 5 g, were purchased from Govt veterinary college Mannuthi, Thrissur, India, were used for the study. The mice were obtained from the stock in breed colony, which was maintained by mating brothers and sisters. They were housed at room temperature of 22°C under 12 hr light/12 hr dark cycle in the animal house. Mice were fed with commercial pellet diet and water *ad libitum* freely throughout the study. All animal procedures were performed after approval from the IAEC (institution of animal ethical committee) and in accordance with the recommendations for the proper care and use of laboratory animals.

3.7.1. DAL - INDUCED ASCITIC ANTITUMOR MODEL[75]

a) ADJUST CELL COUNT TO 1×10^6 cells:

Requirements:

a) Daltons lymphoma ascites (DLA) cells
b) Phosphate buffer saline solutions
c) Haemocytometer
d) Tryphan blue solution (0.4%)

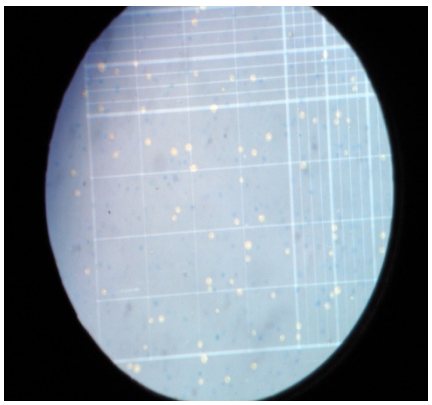
Method:

0.5ml of 0.4% Tryphan blue, 0.3ml of PBS and 0.2ml of cell suspension were mixed and kept aside for 5min and not more than 15min. From this one drop of solution was taken on a neubar chamber and a cover slip is placed. This is placed on

Haemocytometer and the viable and non-viable cells were counted under 10X power. Viable cells doesn't take colour and these cells appear in white colour on blue background Non-viable cells(dead cells) take blue colour and give dark blue shading to the cells, cell count was calculated using formula**.**

Cell count = No. of cells x Dilution factor × volume factor

b) DAL-induced ascitic antitumor model:

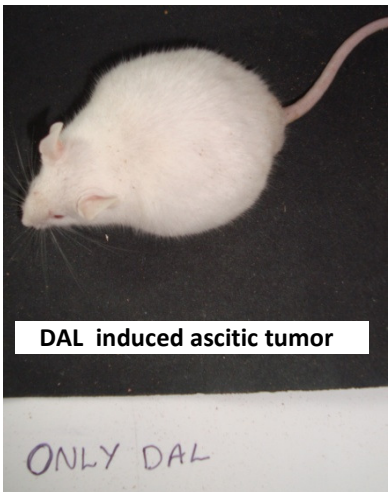


The anti tumor activity of the LEAVES OF *CASSIA FISTULA LINN* was determined by injecting DAL cell suspension (1×10^6 cells per mouse) in to the peritoneal cavity of the animals and was started after 24 hours of the tumor inoculation continued once daily for 14 days as described in the **fig** and the antitumor efficacy of LEAVES OF *CASSIA FISTULA LINN* was compared with that of 5-Fu (10mg/kg, i.p) and DAL control.

3.7.2. DETERMINATION OF BODY WEIGHT AND SURVIVAL TIME

Body weight analysis:

All the mice were weighed for every five days, after tumor inoculation. Average gain in body weight was determined and recorded in table no and fig And a %decrease in body weight were calculated by the formula.



%Decrease in body weight = (Decrease in body weight/initial body weight) ×100

3.7.3. Mean Survival Time (MST):

After induction every day checks all the groups for mortality & record how many days the mouse is survived the mean survival time (MST) and percentage increase in life span (ILS %) was calculated by using the formula.

Mean survival time = [1st Death + Last Death] / 2

ILS (%) = [(Mean survival of treated group/ Mean survival of control group)-1] x100

3.8. DETERMINATION OF HEMATOLOGICAL & IN VIVO-ANTIOXIDANT

3.8.1. Determination of tumor volume:

After 14 days treatment the mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube.

3.8.2. Viable and non-viable cell count:

Requirements:

a) Daltons lymphoma ascites (DLA) cells
b) Phosphate buffer saline solutions
c) Haemocytometer
d) Tryphan blue solution (0.4%)

Method:

After 14 days treatment animals are slightly anaesthetized with diethyl ether from the intraperitoneal cavity of mice take 0.2ml of cell suspension were mixed with 0.5ml of 0.4% typhan blue, 0.3ml of normal saline or PBS and kept aside for 5min and not more than 15min. From this one drop of solution was taken on a neubauer chamber and a cover slip is placed. This is placed on Haemocytometer and the viable and non-viable cells were counted under 10X power Viable cells doesn't take colour and these cells appear in

white colour on blue background Non-viable cells(dead cells) take blue colour and give dark blue shading to the cells, cell count was calculated using formula**.**

Cell count = No. of cells x Dilution factor × volume factor

Preparation of Blood Serum and Tissue Samples For The Bio Chemical Studies:

After 14 days treatment the animals were fasted over night and collect the blood sample and intraperitoneal fluid by mild anaesthetized the mice with diethyl ether and then sacrificed by cervical dislocation.

Blood was collected for estimation of RBC, WBC, Differential cell count , Hb percentage and platelet count and serum was separated from blood cells by centrifugation at about 2000 rpm for 30 min and this serum is used for estimation of SGOT, SGPT, serum creatinine, serum triglycerides, alkaline phosphatase, albumin, total protein values.

Tissues like liver and kidney were removed from the mouse body and tissues were transferred to ice cooled containers. Wiped thoroughly using blotting paper to remove blood and other body fluids then they were washed in normal saline, again wiped desired amounts of dried tissues were used for various biochemical analysis and histopathology studies .

3.8.3. Estimation of Hematological Parameters[76-77]:

A) 3.8.3.1.Enumeration of white blood cells:

The total white blood cells were enumerated according to the method of John (1972)

Reagents:

Turk's fluid (WBC diluting fluid).

Procedure:

Using a white blood cell pipette of haemocytometer, well mixed blood was drawn up to 0.5 mark and WBC diluting fluid was taken up to mark II. The fluid blood mixture was shaken and transferred onto the counting chamber. The cells were allowed to settle to the bottom of the chamber for 2 min. See the fluid does not get dried.

Using 10X or low power objective the WBC's were counted uniformly in the larger corner squares.

The cells were expressed as number of cells $x10^9$/L

B) 3.8.3.2.Enumeration of red blood cells[78]:

Reagents: RBC diluting fluid s

Procedure:

Using a red blood cell pipette of haemocytometer, well mixed blood was drawn up to 0.5 mark and RBC diluting fluid was taken up to mark II. The fluid blood mixture was shaken and transferred onto the counting chamber. The cells were allowed to settle to the bottom of the chamber for 2 min. See the fluid does not get dried. Using 45X or high power objective the RBC's were counted uniformly in the larger corner squares.

The cells were expressed as number of cells $x10^{12}$/Lor$x\ 10^6$ / cu.mm

C) 3.8.3.3.Differential Leukocyte Count[78]:

Differential Leukocyte count was determined by the method of John (1972).

Reagent:

Leishmann's stain: 150mg of powdered leishmann's stain was dissolved in 133ml of acetone free methanol.

Procedure:

A blood film stained with leishmann's stain was examined under oil immersion and the different types of WBCs were identified. The percentage distribution of these cells was then determined. Smears were made from anticoagulant blood specimens and stained with leishmann's stain. The slides were preserved for counting the number of lymphocytes and neutrophils, per 100 cells were noted.

The number of neutrophils was expressed as (%)

D) 3.8.3.4. Estimation of Hemoglobin:

Sahli's acid haematin method[79]:

Principle:

Hemoglobin is converted into acid haematin by the action of HCl. The acid haematin solution is further diluted with distilled water until its colour matches with exactly that of permanent standard of comparator block. The Hb concentration is read directly from the calibration tube.

Requirements:

HCl solution, sahli's Hemoglobinometer, pipette, distilled water.

Procedure:

By using pipette add 0.1 N HCl in the Hemoglobinometer up to the lowest marking. 20µl of blood was drawn up to 20µl in the sahli's pipette. Adjusted the blood column carefully without bubbles. Wiped the excess of blood on the sides of the pipette by using a dry piece of cotton. Blown the blood into the acid solution in the graduated tube, rinsed the pipette well. Mixed the reaction and allow the mixture to stand at room temperature of 10 minutes. Diluted the solution with distilled water by adding few drops of water carefully and by mixing the reaction mixture until the colour matches the colour in the comparator. The lower meniscus of the fluid was noted and reading was noted in g/100ml.[79]

3.9. ESTIMATION OF SERUM BIOCHEMICAL PARAMETERS:

SGOT, SGPT was estimated using whole blood. All the above biochemical parameters were estimated using semi-auto analyzer (Photometer 5010 $_{V5+}$, Germany) with enzymatic kits procured from Primal Healthcare limited, Lab Diagnostic Division, Mumbai, India.)

3.9.1. ESTIMATION OF SGOT:

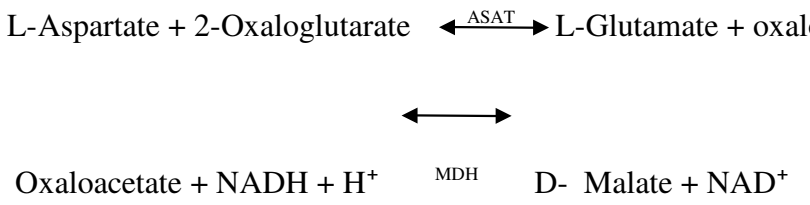
Serum glutamate oxaloacetate transaminase (SGOT)[80]:

Principle:

Alanine aminotransferase (ALAT) and aspartate amino transferase (ASAT) are the most important of a group of enzymes of aminotransferase. These enzymes act as catalyst in conversion of α-keto acids in to amino acids by transfer of amino groups.

Increased levels of ALAT is found in the hepatobillary disease condition where as increased ASAT levels occur in damaged conditions of heart and skeletal muscles well as liver parenchyma. Parallel measurement of ALAT and ASAT is therefore applied to distinguish liver from heart or skeletal muscle damages

The ASAT/ALAT ratio is used from differential diagnosis of liver diseases



Method:

Optimized UV- test according to IFCC (International Federation of Clinical Chemistry and Laboratory Medicine).

Table 3.5. Shows Reagents of SGOT in the kit

Reagent-1	**concentration**
TRIS Ph 7.8	**80 mmol/l**
MDH (malatedehydrognase)**240 mmol/l**	≥600 U/l

L- Aspartate	
LDH(lactate dehydrogenase)	≥600 U/l
Reagent-2	
2-Oxaloglutarate	12 mmol
NADH	0.18 mmol
Good buffer pH 9.6	0.7 mmol/l
Pyridoxal-5-Phosphate	0.09 mmol/l

Assay procedure:

a) Mixed 800 µl of reagent-1 with 200 µl of reagent-2 in a 5 ml test tube.

b) To this, added 100 µl of serum.

c) Mixed well and took the reading immediately.

Normal range : <37u/l.

B) 3.9.2. ESTIMATION OF SGPT: Serum glutamate pyruvate transaminase (SGPT)[81]:

Principle:

L-Alanine +2-oxoglutarate $\xleftrightarrow{ALAT}$ L-Glutamate + Pyruvate

$\Updownarrow$

Pyruvate + NADH + H^+ D-Lactate + NAD^+

Addition of pyridoxal-5-phosphate (P-5-P) stabilizes the transaminases and avoids falsely low values in samples containing insufficient endogenous P-5-P, eg. from patients with myocardial infarction, liver diseases and intensive care patients.

Method:

Kinetic UV test,according to the international federation of clinical chemistry and laboratory medicine (IFCC).

Table 3.6.Shows reagents of **SGPT in the kit**

Reagent-1	**Concentration**
TRIS PH 7.5	**100 mmol/l**
L-Alanine	500 mmol/l
LDH (lactate dehydrogenase)	≥1200 U/l
Reagent-2	**Concentration**
2-Oxoglutarate	15mmol/l
NADH	0.18 mmol/l
Good's buffer PH 9.6	0.7mmol/l
Pyridoxal -5-Phosphate	0.09 mmol/l

Assay procedure:

Mix 800 µl of reagent-1 with 200 µl of reagent -2 in a 5 ml test tube.

a) To this, added 100 µl of serum.

b) Mixed well and took the reading immediately.

Normal range: <41u/l.

3.10. IN VIVO ANTI OXIDANT STUDIES[82]

Preparation of tissue homogenate

The tissue were weighed and 10% tissue homogenate was prepared with 0.025 M Tris –HCl buffer, Ph 7.5.After centrifugation at 10,000 ×g for 10 min, the clear supernatant was used to measure thiobarbituric acid reactive substances(TBARS).

For the estimation of non-enzymic and enzymic antioxidants, tissue was minced and homogenized (10% w/v) in 0.1 M phosphate buffer (Ph 7.0) and centrifuged for 10 min and the resulting supernatant was used for enzyme assays.

A) 3.10.1 Enzymatic antioxidant activity:-

I. 3.10.1.1.Estimation of Superoxide Dismutase (SOD) activity :-

Reagents:

1. Adrenaline
2. Carbonate buffer (pH10.2),
3. 0.1Mm EDTA

The activity of superoxide dismutase (SOD) was assayed by the method of Kakkar et al based on the oxidation of epinephrine adrenochrome transition by enzyme. The post-mitochondrial suspension of mice liver (0.5ml) was diluted with distilled water (0.5). To this chilled ethanol (0.25ml) and chloroform (0.15ml) were added. The mixture was shaken for 1 min and centrifuged at 2000 × g for 10 min.The PMS (0.5ml) was

added with PBS buffer (Ph 7.2; 1.5ml).The reaction initiated by the addition of epinephrine(0.4ml) and change in optical density (O.D.,min-1) was measured at 470 nm.SOD activity was expressed as U/mg of tissue.Change in O.D (min-1) at 50% inhibition to adrenochrome transition by the enzyme was taken as one enzyme unit.

II. 3.10.1.2.Estimation of Catalase (CAT) activity:-[83]

Reagents:

1. Dichromate/acetic acid reagent (5% solution of potassium dichromate in acetic acid at 1:3 ratios)

2. 0.01 M Phosphate buffer, pH 7.0

3. 0.2 M Hydrogen peroxide

Catalase(CAT) was estimated by the method of Sinha (1972).The reaction mixture (1.5ml vol) contained 1.0 ml of 0.01 M phosphate buffer (Ph7.0) 0.1 ml of tissue homogenate and 0.4 ml of 2M H2O2. The reaction was stopped by the addition of 2.0 ml dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio).Then thebabsorbance was measured at 530 nm; CAT activity was expressed as µM of H2O2 consumed/min/mg protein

III. 3.10.1.3. Estimation of Glutathione peroxidase (GPx) activity:-[84]
Reagents:

1. 0.32 M Phosphate buffer, pH 7.0

2. 0.8 mM EDTA

3. 10 mM Sodium azide

4. 3 mM reduced glutathione

5. 2.5 mM H_2O_2

6. 10% TCA

7. 0.3 M Disodium hydrogen phosphate

8. DTNB solution (40 mg of DTNB in 100 ml of 1% sodium citrate)

Procedure:

Glutathione peroxidase (GPx) was measured by the method described by Rotruck et al.(1973).Briefly, the reaction mixture contained 0.2 ml 0.4 M phosphate buffer (Ph 7.0),0.1 ml 10m M sodium azide,0.2 ml tissue homogenized in 0.4 M ,phosphate buffer,Ph 7.0,0.2 ml tissue homogenized in 0.4 M,phosphate buffer ,Ph 7.0,0.2 ml reduced glutathione ,and 0.1 ml 0.2 mM hydrogen peroxide. The contents were incubated for 10 min at 37 ^{0}c,0.4 ml 10% TCA was added to stop the reaction and centrifuged at 3200 × g for 20 min.The supernatant was assayed for glutathione content using Ellman's reagent (19.8 mg 5,5'-dithiobisnitrobenzoic acid (DTNB) in 100 ml 0.1% sodium nitrate). The activities were expressed as µg of GSH consumed/ min/mg protein.

B) 3.10.2. Non enzymatic anti oxidant activity

I. 3.10.2.1. Estimation of reduced glutathione(GSH) activity:-[85]

Reagents:

1. 10% TCA

2. 0.6 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in 0.2 M sodium phosphate

3. 0.2 M Phosphate buffer, pH 8.0

Reduced glutathione (GSH) was measured by the method of Ellman (1959).The PMS of rat liver (720 µl) and 5% TCA were mixed to precipitate the protein content of the supernatant.After centrifugation at 10,000 × g for 5 min, the supernatant was taken.

DTNB (5,5'-dithio-bis(2-nitrobenzoic acid) Ellman's reagent was added to it and the absorbance was measured at 412 nm. A standard graph was drawn using different concentration of standard GSH solution.GSH contents were calculated in the PMS of rat liver.

II. 3.10.2.2. Estimation of Lipid Peroxidation of Rat Liver and Kidneys[86]

Reagents:

1. Thiobarbituric acid 0.37%

2. 0.25 N HCl

3 . 15% TCA

Lipid peroxidation in liver and kidney was estimated calorimetrically by measuring thiobarbituric acid reactive substances (TBARS) using the method of Fraga et al.(1988). In brief,0.1 ml of tissue homogenate was treated with 2 ml of TBA-trichloroacetic acid –HCl reagent (0.37% TBA,0.25 M HCl and 15% TCA, 1:1:1 ratio), placed for 15 min in a water bath and then cooled and centrifuged at 3500 ×g for 10 min at room temperature, the absorbance of clear supernatant was measured at 535 nm against a reference blank. Values were expressed as Mm/100 g tissue.

C) 3.10.2.3. Estimation of proteins[87]

Principle

Procedure described by Lowery et al(1951) was used for protein estimation. The method is based on the biuret reaction, formation of a protein-copper complex and reduction of phosphor molybdo tungstate reagent (Folin-ciocalteu phenol reagent) by tyrosine and tryptophan residues of protein to form a coloured product.

Reagents:

Solution A: 1ml CuSO4 5H2O (1%) + 1ml sodium potassium tarterate (2%) + 98 ml 2% Na2CO3 in 0.1N NaOH.

Solution B: Folin Ciocalteu reagent and distilled water mixed in 1:1 ratio just before use.

Procedure:

0.01 ml of tissue homogenate (2.5%) was diluted to 1.2 ml and mixed with 6 ml of solution A. The mixtures was incubated at room temperature for 10 min and add 0.3 ml solution B was added, mixed immediately and kept at room temperature for 30 min. optical density was taken at 750 nm. The amount of protein was calculated from the standard curve of bovine serum albumin (BSA).

D) 3.10.2.4. Estimation of ascorbic acid vitamin C[88]

The level of ascorbic acid was estimated by the method of Omaye *et al.*1979).

Reagents

1. 5% TCA
2. DTC reagent 3 g of 2,4-dinitrophenyl hydrazine, 0.4 g of thiourea and 0.05 g of copper sulphate were dissolved in 100 ml of 9 N sulphuric acid)
3. 65% sulphuric acid
4. Ascorbic acid

Procedure

To 0.5 ml of homogenate, 0.5 ml of water and 1 ml of TCA were added, mixed thoroughly and centrifuged. To 1 ml of the supernatant, 0.2 ml of DTC reagent was added and incubated at 37°C for 3 hrs. Then 1.5 ml of sulphuric acid was added, mixed well and

the solutions were allowed to stand at room temperature for another 30 min. The colour developed was read at 520 nm.

The level of ascorbic acid was expressed as μg/mg protein

E) 3.10.2.5. Estimation of vitamin E[88]

The level of vitamin E was estimated by the method of Desai 1984).

Reagents

1. Ethanol
2. Petroleum ether
3. 0.2% 4,6-diphenyl-1,10-phenanthroline in ethanol
4. 0.001 M Ferric chloride in ethanol
5. 0.001 M o-phosphoric acid in ethanol
6. α-Tocopherol acetate

Procedure

To 1 ml of homogenate, 1 ml of ethanol was added and thoroughly mixed. Then 3 ml of petroleum ether was added, shaken rapidly and centrifuged. 2 ml of supernatant was taken and evaporated to dryness. To this 0.2 ml of bathophenanthroline was added. The assay mixture was protected from light and 0.2 ml of ferric chloride was added followed by 0.2 ml of o-phosphoric acid. The total volume was made up to 3 ml with ethanol. The colour developed was read at 530 nm.

The level of vitamin E was expressed as μg/mg protein.

3.11. HISTOPHATOLOGICAL TECHINIQUES:

Histopathology is the microscopical study of tissues for pathological alterations. This involves collection of morbid tissues from biopsy or necropsy, fixation, preparation of sections, staining and microscopical examination.

Collection of materials:

Thin pieces of 3 to 5 mm, thickness are collected from tissues showing gross morbid changes along with normal tissue.

Fixation:

Keeping the tissue in Fixative for 24-48 hours at room temperature

a) Serves to harden the tissues by coagulating the cell protein,
b) Prevents autolysis,
c) Preserves the structure of the tissue, and
d) Prevents shrinkage: The volume of the fixative added is 10times the volume of the tissues.
   Common Fixatives:10% Formalin.

Haematoxylin and eosin method of staining:

Deparaffinise the section by xylol 5 to 10 minutes and remove xylol by absolute alcohol, then wash in tap water. Stain with haematoxylin for 3-4 minutes and wash in tap water. Allow the sections in tap water 5-10 min and wash in tap water. Counter stain with 0.5% until section appears light pink(15 to 30seconds), then wash in tap water. Blot and dehydrate in alcohol. Clear with xylol (15 to 30 seconds). Mount in Canada balsam or DPX Moutant. Keep slide dry and remove air bubbles.

4. RESULTS

Phytochemical studies of Methanolic extracts of leaves of *Cassia fistula linn.*

4.1.1 Preliminary Phytochemical Tests

Table 4.1 Shows preliminary qualitative chemical tests of Cassia fistula linn

S.N.	Phytochemical constituents	*Cassia fistula linn*
1.	Carbohydrates	+ve
2.	Alkaloids	+ve
3.	Steroids & sterols	+ve
4.	Glycosides	+ve
5.	Saponins	+ve
6.	Flavonoids	+ve
7.	Tannins & phenolic compound	+ve
8.	Proteins & amino acids	-ve

4.2. INVITRO ANTI OXIDANT STUDY

4.2.1. DETERMINED BY MTT ASSAY AGAINST HELA CELL LINES:

Table 4.2. Shows of the Toxicity in *Cassia fistula* determined by MTT assay against HELA cell lines

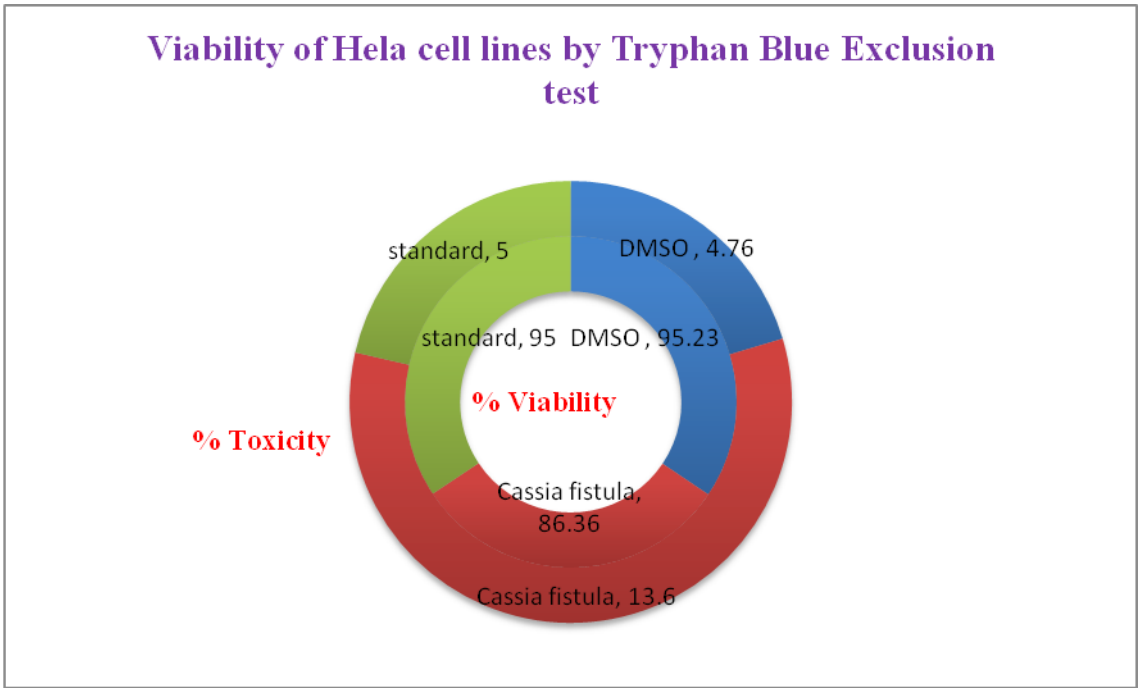
Groups	Concentration	Percentage Toxicity
DMSO		7.90 ± 0.20
Standard	25 mg/ml	15.50 ± 0.53**
	50 mg/ml	12.33 ± 0.42**
	100 mg/ml	14.27 ± 0.17**
Cassia fistula	25 mg/ml	11.90 ± 0.40**
	50 mg/ml	13.60 ± 0.20**
	100 mg/ml	11.07 ± 0.17**

Values are expressed as mean ± SEM. **p<0.01 vs DMSO (Oneway ANOVA followed by Dunnett's test)

4.2.2. TRYPHAN BLUE DYE EXCLUSION TEST

Table 4.3. Shows of the Viability of Hela cell lines by *Cassia fistula* determined on Tryphan Blue Exclusion test

	Concentration	% Viability	% Toxicity
DMSO		95.23	4.76
Cassia fistula	100 mg/ml	86.36	13.6
Standard	100 mg/ml	95	5



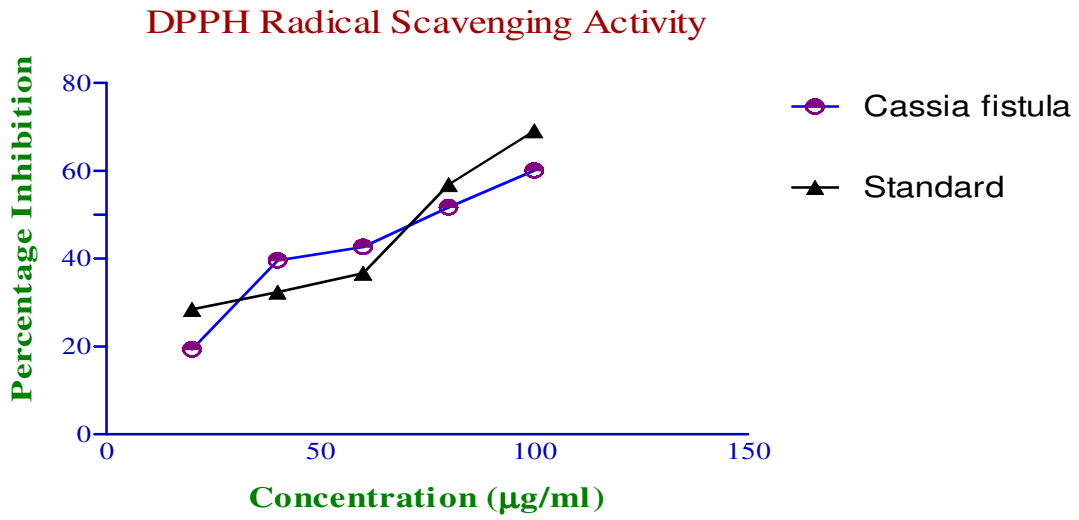
4.3. INVITRO ANTI OXIDANT STUDY

4.3.1. DPPH Radical Scavenging Activity:

Table 4 .4. Shows % inhibition of DPPH radical scavenging activity of Methanolic extracts of leaves of *Cassia fistula linn*.

CONTROL: 0.4335

Serial number	Concentration (µg/ml)	% of activity			
		Cassia fistula		Standard	
		MEAN	SEM	MEAN	SEM
1	20.000	19.37563	0.3221953	28.44137	0.322195
2	40.000	39.62937	1.146634	32.33987	1.146635
3	60.000	42.69743	0.9489966	36.69973	0.9489955
4	80.000	51.72472	0.8438241	56.8689	0.8438246
5	100.000	60.09842	1.103273	69.09496	1.103276
		IC_{50} =71.29		IC_{50} =66.26	

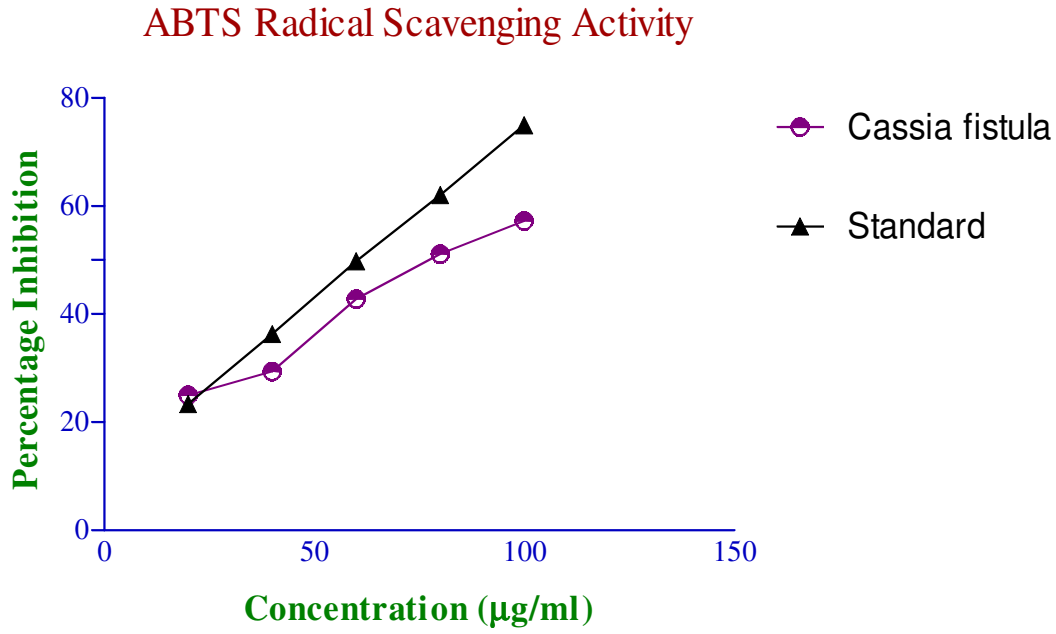


4.3.2 ABTS: Radical Scavenging Activity:

Table4.5. Shows % inhibition of ABTS radical scavenging activity of Methanolic extracts of leaves of *Cassia fistula linn.*

CONTROL: 0.8615

Serial number	Concentration (µg/ml)	% of activity			
		Cassia fistula		Standard	
		MEAN	SEM	MEAN	SEM
1	20.000	24.94409	0.1621264	23.39253	0.1410932
2	40.000	29.355	0.5769777	36.29251	0.442966
3	60.000	42.77346	0.4775271	49.74192	0.5913936
4	80.000	51.11163	0.4246065	62.00735	0.4365487
5	100.000	57.24047	0.5551583	74.9615	0.3762644
		IC_{50} =79.05		IC_{50} =54.99	

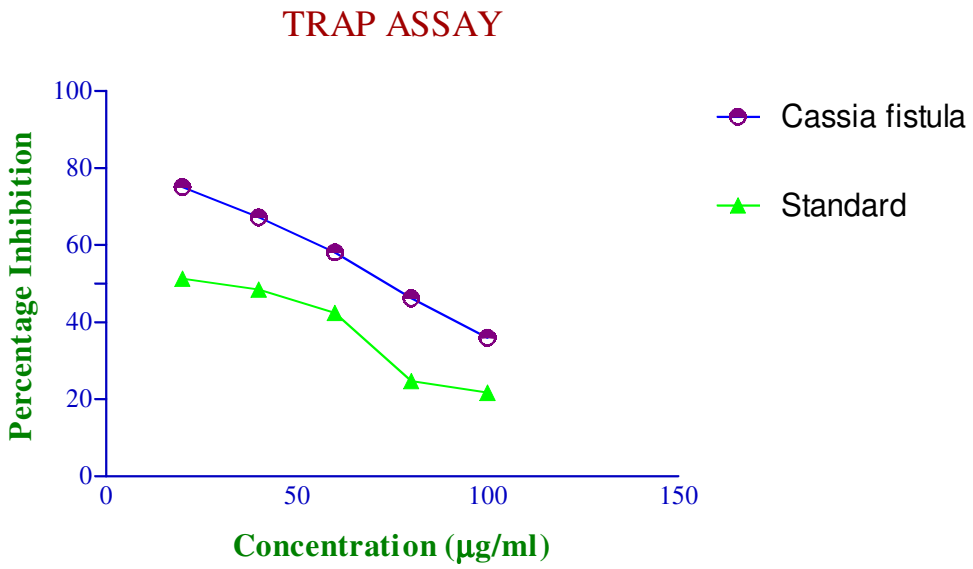


4.3.3 TRAP: Radical Scavenging Activity:

Table4.6. Shows % inhibition of TRAP radical scavenging activity of Methanolic extracts of leaves of *Cassia fistula linn.*

CONTROL: 0.9615

Serial number	Concentration (µg/ml)	% of activity			
		Cassia fistula		Standard	
		MEAN	SEM	MEAN	SEM
1	20.000	75.04178	0.1264189	51.28376	0.1452642
2	40.000	67.23453	0.3968955	48.45485	0.5169694
3	60.000	58.1099	0.5298852	42.3498	0.427863
4	80.000	46.24996	0.3911458	24.71416	0.3804448
5	100.000	35.94315	0.3371318	21.68764	0.4974199
		IC_{50} =69.28		IC_{50} =27.40	

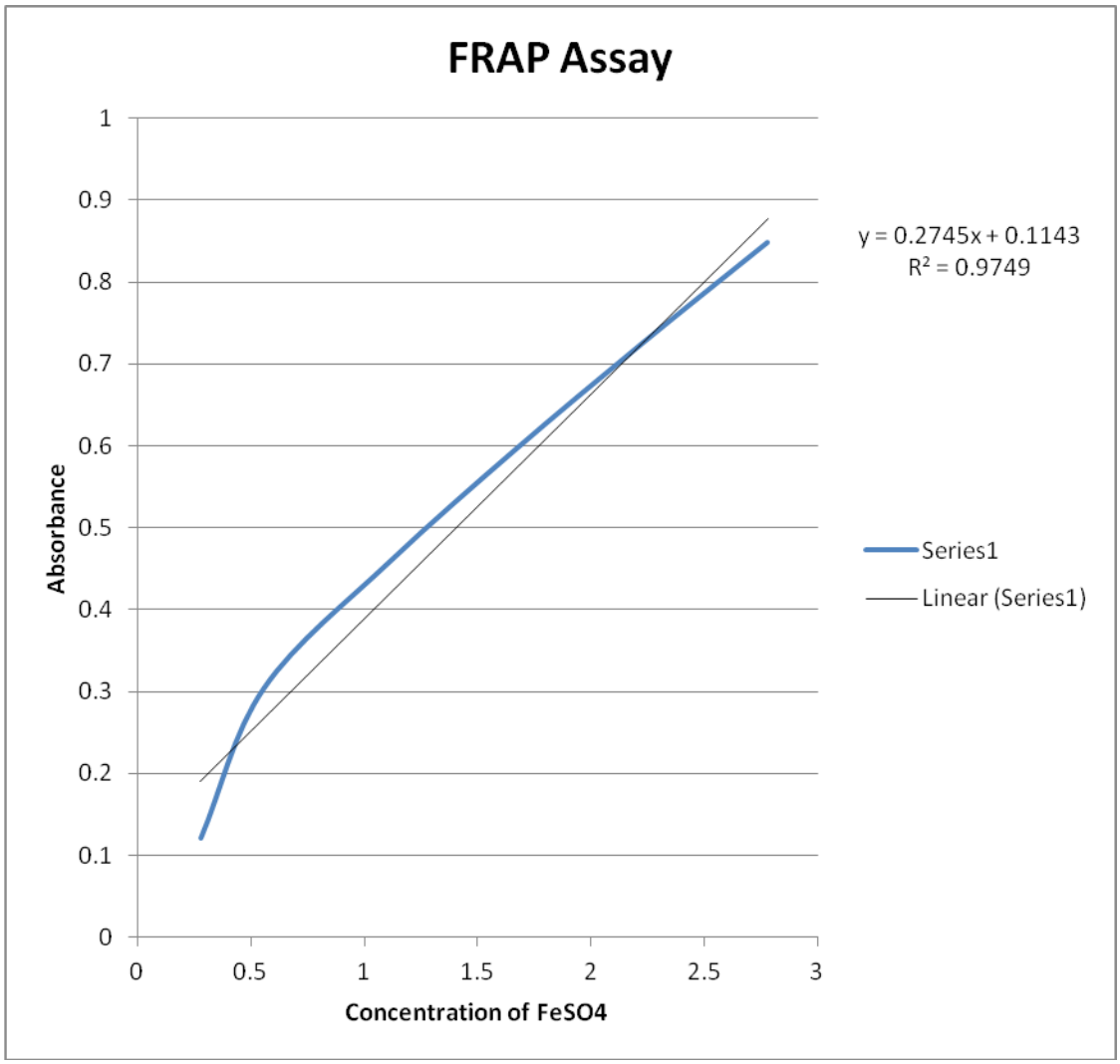


4.3.4 FRAP: Radical Scavenging Activity:

Table4.7. Shows % inhibition of FRAP radical scavenging activity of Methanolic extracts of leaves of *Cassia fistula linn.*

Sample	Concentration	Absorbance		
Cassia fistula	10	0.9154	0.9122	0.9126
	20	1.0554	1.0526	1.0548
	30	1.2274	1.2268	1.2237
	40	1.3145	1.3124	1.3187
	50	1.3684	1.3626	1.3625
Standard	0.1	0.1214	0.1216	0.1212
	0.2	0.3041	0.3045	0.3037
	0.4	0.4586	0.4592	0.4580
	0.6	0.5955	0.5957	0.5956
	0.8	0.7243	0.7250	0.7236
	1.0	0.8476	0.8482	0.8479

Concentration	Absorbance (mean ± SEM)	
10	0.9134	0.001006662
20	1.0543	0.0008511411
30	1.2260	0.001146461
40	1.3152	0.001852018
50	1.3645	0.00195022



Extract 1

50µg of extract is equivalent to 4.56 µg of standard

10.97 mg of Extract is equivalent to 1 mg of Standard

4.4. DAL INDUCED ACITIC TUMOR:

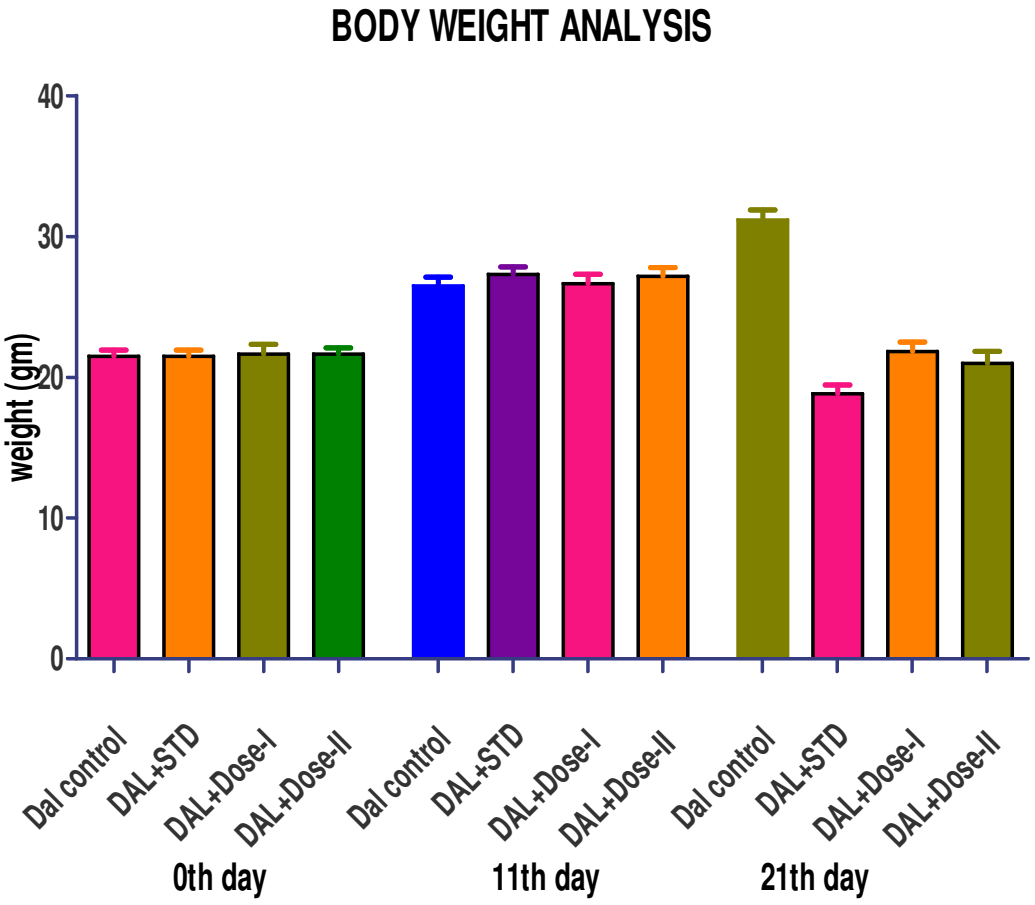
4.4.1. BODY WEIGHT ANALYSIS:

Effect of Methanolic extract of Leaves of *Cassia fistula linn* on body weight of mice challenged with Daltons lymphoma ascetic cells.

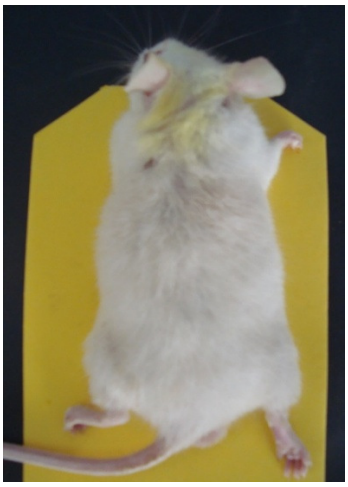
Table 4.8. Shows body weight of various groups for every 10 days

EXPERIMENTAL GROUPS	Body weight (gm)			Decrease in body weight from 11th day to 20th day(gm)	%decrease in body weight
	0 day	11th day	20th day		
DAL CONTROL	21.50±0.428	26.500±0.6191	31.16667±0.70	---------- --	---------- ---
DAL+STD	21.50±0.428	27.33333±0.49	18.83333±0.60	8.5	39.53
DAL+DOSE-I	21.67±0.666	26.66667±0.66	21.83333±0.65	4.8	22.22
DAL+DOSE-II	21.67±0.421	27.16667±0.60	21.000±0.8563	6.1	28.46

Values are expressed as the Mean ± S.E.M., (n = 6) on day 14 of the experiment; Statistical significance (p) calculated by one way ANOVA followed by Dunnett's [a]P<0.01 calculated by comparing treated group with 5- fu standard group.

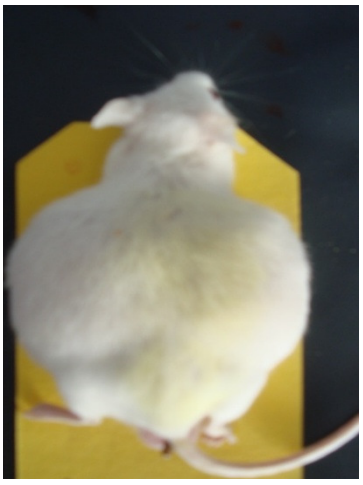

BODY WEIGHT ANALYSIS
weight (gm)
40
30
20
10
0
Dal control
DAL+STD
DAL+Dose-I
DAL+Dose-II
Dal control
DAL+STD
DAL+Dose-I
DAL+Dose-II
Dal control
DAL+STD
DAL+Dose-I
DAL+Dose-II
0th day
11th day
21th day

EFFECT OF METHANOLIC EXTRACTS OF LEAVES OF *Cassia fistula linn* ON BODY WEIGHT ANALYSIS

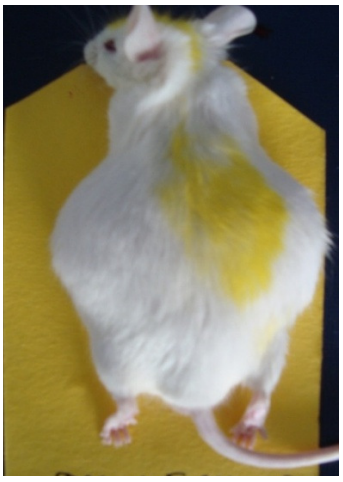


ONLY VEHICLE

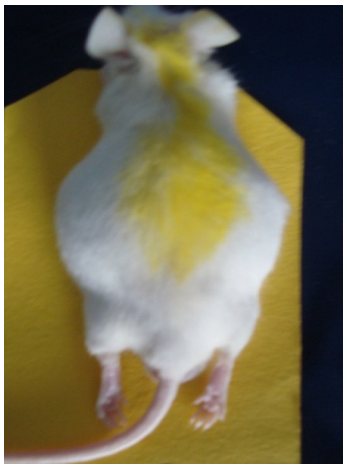
ONLY DAL



DAL + 5 FLUROURACIL 20mg/kg



DAL + *CASSIA FISTULA* 250mg/kg



DAL + *CASSIA FISTULA* 500mg/kg

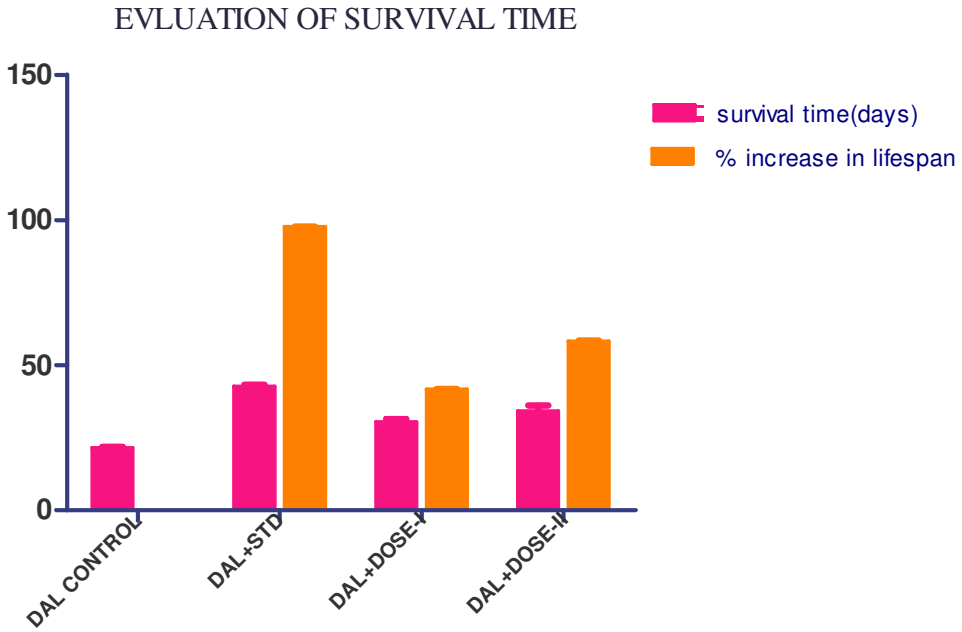
4.4.2. MEAN SURVIVAL TIME (MST) :

Effect of Methanolic extract of Leaves of *Cassia fistula linn* on survival time of mice challenged with Daltons lymphoma ascetic cells.

Table 4.9.Shows mean survival time and percentage increase in life span various group

Experimental groups	Mean survival time in days	%ILS
DAL CONTROL	21.50±0.43	-
DAL+STD	42.50±0.76a	97.674
DAL+DOSE-I	30.33333±1.021981[a]	41.0853
DAL+DOSE-II	34.16667±1.922094[a]	58.9147

Values are expressed as the mean ± S.E.M.; Statistical significance (p) calculated by one way ANOVA followed by dunnett's [a]$P < 0.05$, NS – non significant calculated by comparing treated group with DAL control group.

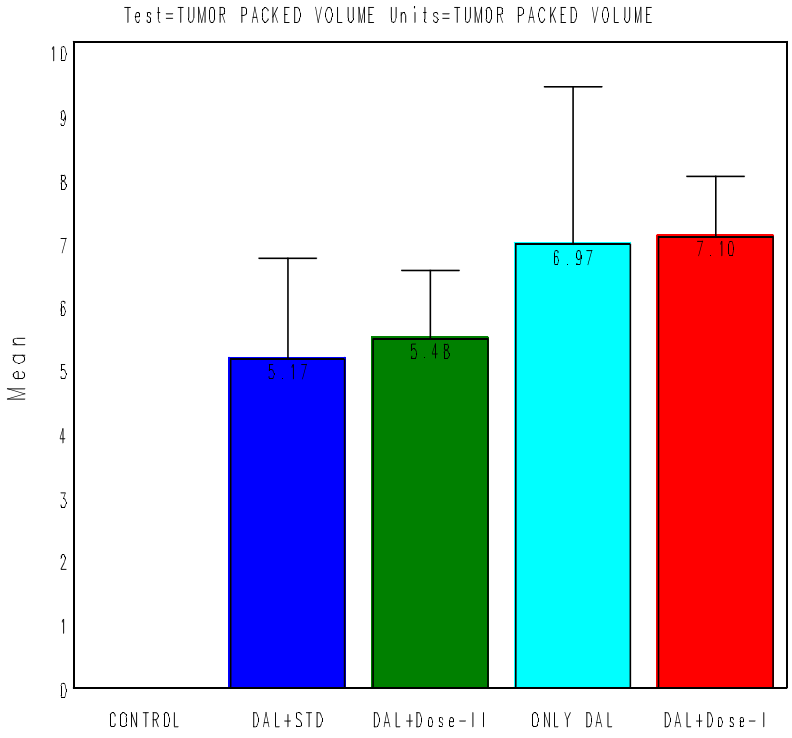


4.5. **EFFECT ON TUMOR PACKED CELL VOLUME and TUMOR VOLUME on Methanolic extracts of leaves of *Cassia fistula linn***

Table 4.10. Shows tumor volume of experimental groups.

Test	CONTROL	DAL_Dose_I	DAL_Dose_II	DAL_STD	ONLY_DAL
TUMOR PACKED VOLUME	0.00±0.00a	7.10±0.890	5.48±1.025	5.17±1.508	6.97±2.368
TUMOR VOLUME	0.00±0.00a	20.67±5.785	14.67±3.266a	10.00±4.64a	22.83±4.401

Values are expressed as the mean ± S.E.M.; Statistical significance (p) calculated by one way ANOVA followed by Dunnett's [a]*P*< 0.05 , NS – non significant calculated by comparing treated group with DAL control group.

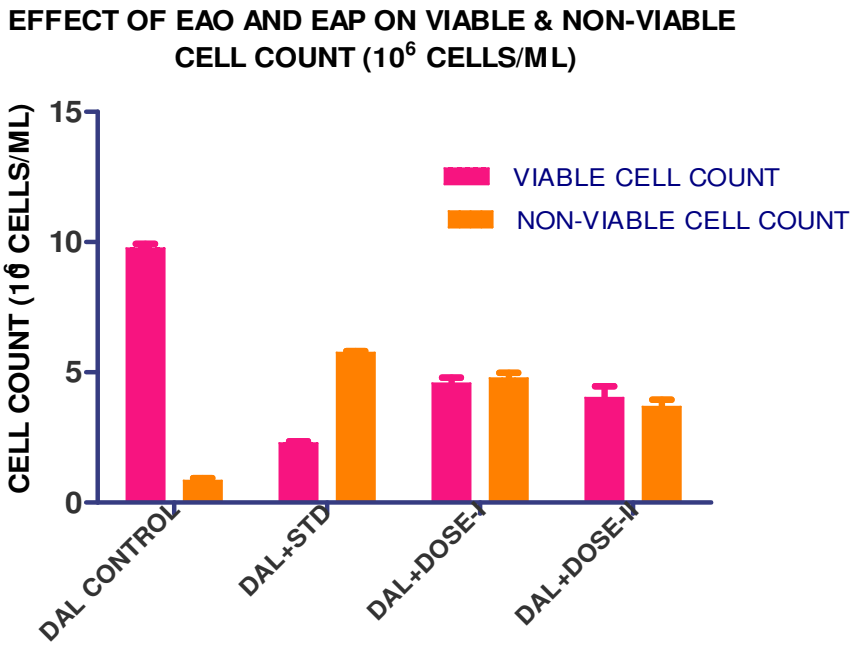


4.6. EFFECT OF METHANOLIC EXTRACTS OF LEAVES OF *Cassia fistula linn* ON VIABLE AND NON-VIABLE CELL COUNT

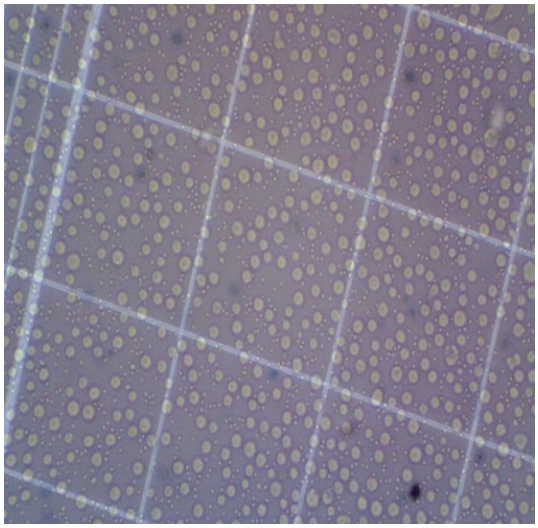
Table 4.11.Shows VIABLE AND NON-VIABLE CELL COUNT

EXPERIMENTAL GROUPS	DAL CONTROL	DAL+STD	DAL+Dose-I	DAL+Dose-II
Viable cell count	9.77±0.17	2.28±0.07[a]	4.583333±0.2166667[a]	4.033333±0.43122[a]
Non-viable cell count	0.84±0.10	5.75±0.07[a]	4.783333±0.1973435[a]	3.683333±0.2676025[a]

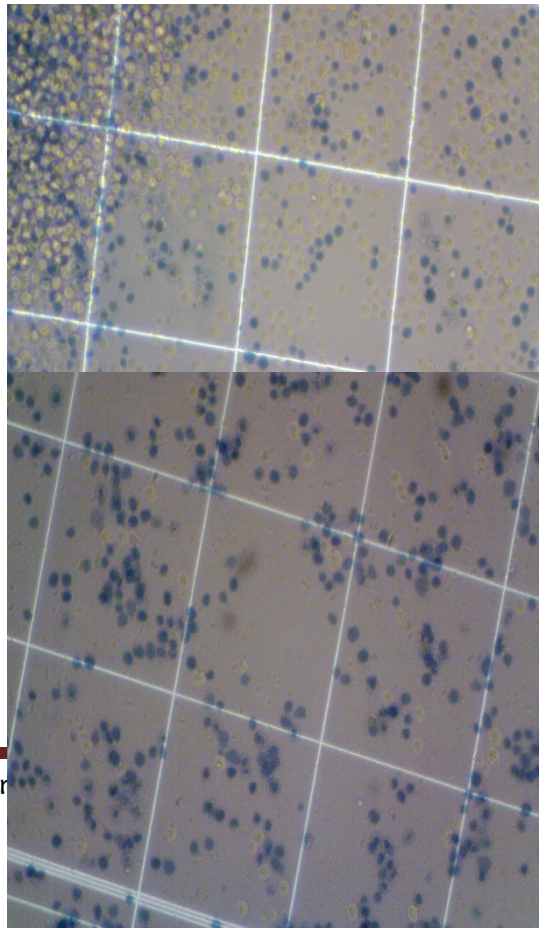
Values are expressed as the mean ± S.E.M., (n = 6) on day 14 of the experiment; Statistical significance (p) calculated by one way ANOVA followed by Dunnett's [a]*P*< 0.05 , NS – non significant calculated by comparing treated group with DAL control group.



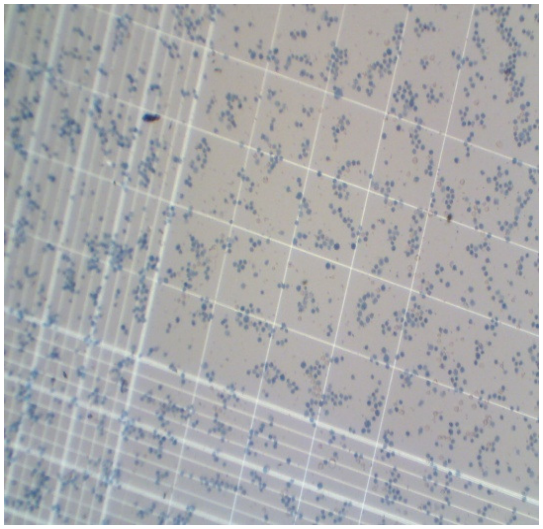
EFFECT OF METHANOLIC EXTRACTS OF LEAVES OF *Cassia fistula linn* ON VIABLE AND NON-VIABLE CELL COUNT



ONLY DAL



DAL + 5 FLUROURACIL



DAL + *Cassia fistula* 250mg/kg

DAL + *Cassia fistula* 500mg/kg

4.7. Effect of Methanolic extract of Leaves *of Cassia fistula linn* on hematological parameters in mice challenged with Daltons lymphoma ascetic cells.

Test	Units	CONTROL	DAL Dos _I	DAL Dos II	DAL+STD	ONLY DAL
ESONOPHIL	%	2.00±0.632a	5.83±2.137	5.50±0.548	3.67±1.633a	7.00±1.414
HB	gdl	14.97±074a	13.28±0.71 a	11.35±0.979	13.04±1.447 a	10.65±1.063
LYMPHOCYTE	%	93.33±2.53a	84.33±4.74 a	87.33±1.751a	84.83±2.639 a	74.83±4.119
MCH	Pictogra mscell	16.90±1.81a	14.85±2.19 a	15.05±1.475a	16.25±2.130 a	21.37±2.002
MONOCYTE	%	3.17±1.169a	7.67±2.944	4.50±1.378a	4.83±1.722a	10.33±2.160
NEUTROPHIL	%	1.33±0.516a	2.17±1.472 a	2.50±0.837a	6.67±2.160	8.17±2.714
PCV	%	50.25±1.58a	40.20±5.81 a	37.75±3.109a	42.53±2.982 a	30.40±4.769

Test	Units	CONTROL	DAL Dos _I	DAL Dos II	DAL+STD	ONLY DAL
RBC	1*10000 00MM3	9.19±0.349a	7.74±0.640 a	7.42±0.681a	7.50±0.753a	5.14±0.687
WBC	1*1000 MM3	16.21±8.38a	21.47±5.90 a	22.82±9.53a	23.27±11.02 a	38.83±5.695

Values are expressed as the mean ± S.E.M., (n = 6) on day 13 of the experiment; [a]*P*<0.05, [B]P<0.01, and [C]P<0.001 between normal and tumor control [a]*P*<0.05, [B]P<0.01, and [C]P<0.001 between normal and tumor control and treated groups and ns - non significant.

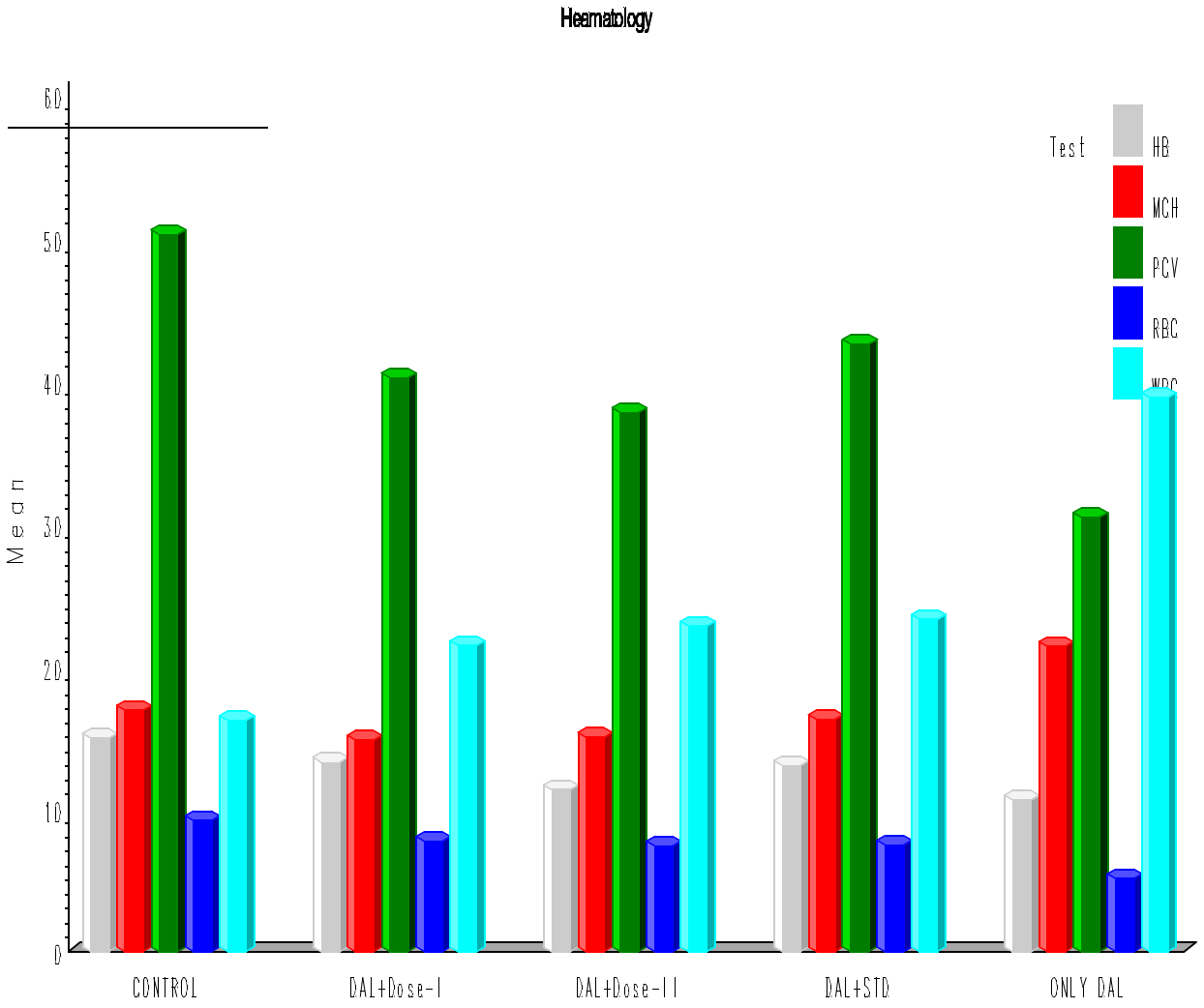


Figure ; **RBC, WBC, HB, PCV, NEUTROPHIL, ESONOPHIL, LYMPHOCYTE MONOCYTE MCH.**

4.8. Effect of Methanolic extract of Leaves of *Cassia fistula linn* serum biochemical parameters in mice challenged with Daltons lymphoma ascetic cells.

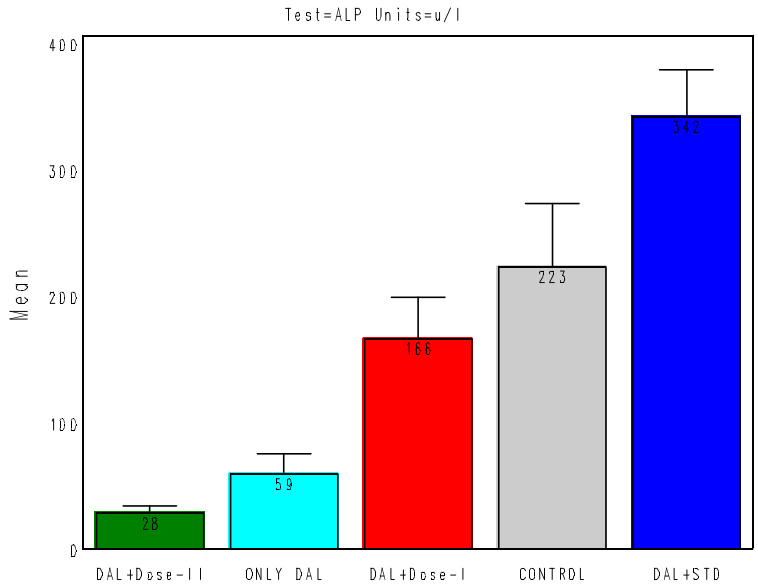
ALKALINE PHOSPHATASE (ALP):

Table 4.12.Shows Serum in ALP levels.

Test	Units	CONTROL	DAL +DOSE-I	DAL + DOSE-II	DAL + STD	ONLY DAL
ALP	u/l	223.00±47.9 a	165.83±31.70 8a	27.87±5.41 5	342.00±35.54 2a	58.50±15.66 8

Values are expressed as the mean ± S.E.M., (n = 6) on day 14 of the experiment; [a]*P*<0.05, [b]P<0.01, and [c]P<0.001 between normal and tumor control [d]*P*<0.05, [e]P<0.01, and [f]P<0.001 between tumor control and treated groups and ns- non significant.

ALKALINE PHOSPHATASE (ALP)



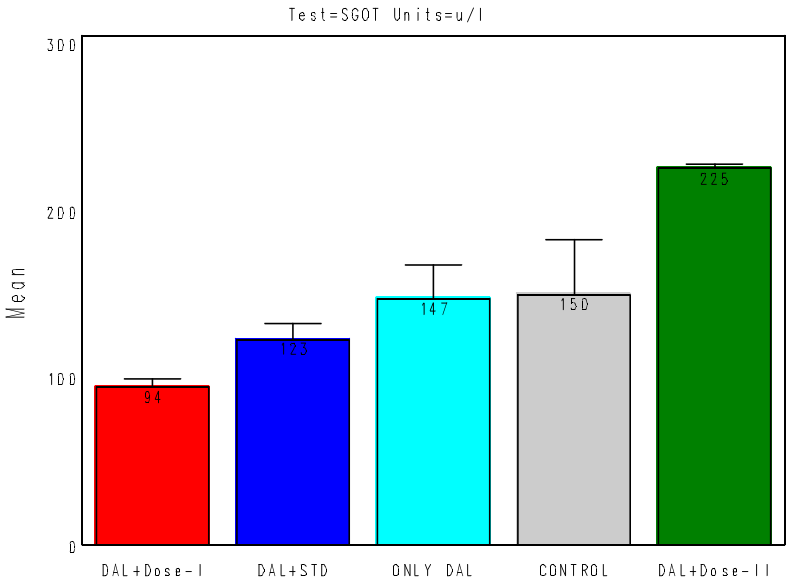
4.9. SERUM GLUTAMATE OXALOACETATE TRANSAMINASE (SGOT):

Table 4.13.Shows Serum in SGOT levels.

Test	Units	CONTROL	DAL +DOSE-I	DAL + DOSE-II	DAL + STD	ONLY DAL
SGOT	u/l	149.50±31.348	94.33±4.66a	225.17±2.317a	122.83±9.065	147.33±19.222

Values are expressed as the mean ± S.E.M., (n = 6) on day 14 of the experiment; [a]*P*<0.05, [b]P<0.01, and [c]P<0.001 between normal and tumor control [d]*P*<0.05, [e]P<0.01, and [f]P<0.001 between tumor control and treated groups and ns - non significant.

GLUTAMATE OXALOACETATE TRANSAMINASE (SGOT)



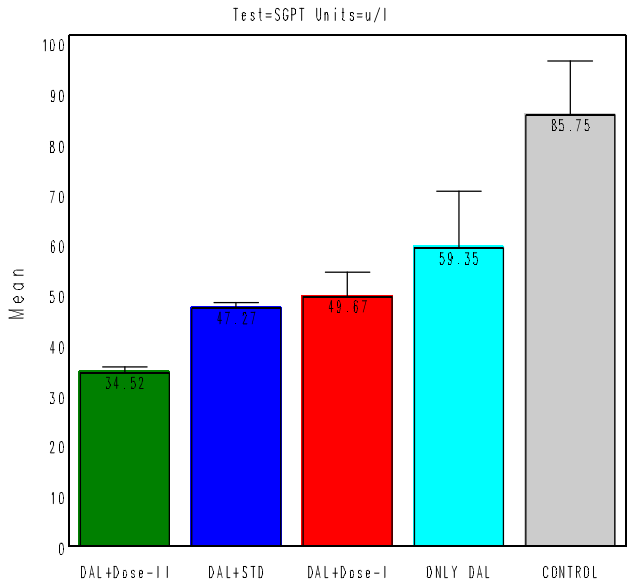
4.10. SERUM GLUTAMATE PYRUVATE TRANSAMINASE (SGPT):

Table 4.14:Shows Serum in SGPT levels.

Test	Units	CONTROL	DAL + DOSE-I	DAL + DOSE-II	DAL + STD	ONLY DAL
SGPT	u/l	85.75±10.36a	49.67±4.59	34.52±1.059a	47.27±1.106a	59.35±10.706

Values are expressed as the mean ± S.E.M., (n = 6) on day 14 of the experiment; [a]*P*<0.05, [b]P<0.01, and [c]P<0.001 between normal and tumor control [d]*P*<0.05, [e]P<0.01, and [f]P<0.001 between tumor control and treated groups and ns- non significant.

GLUTAMATE PYRUVATE TRANSAMINASE (SGPT)



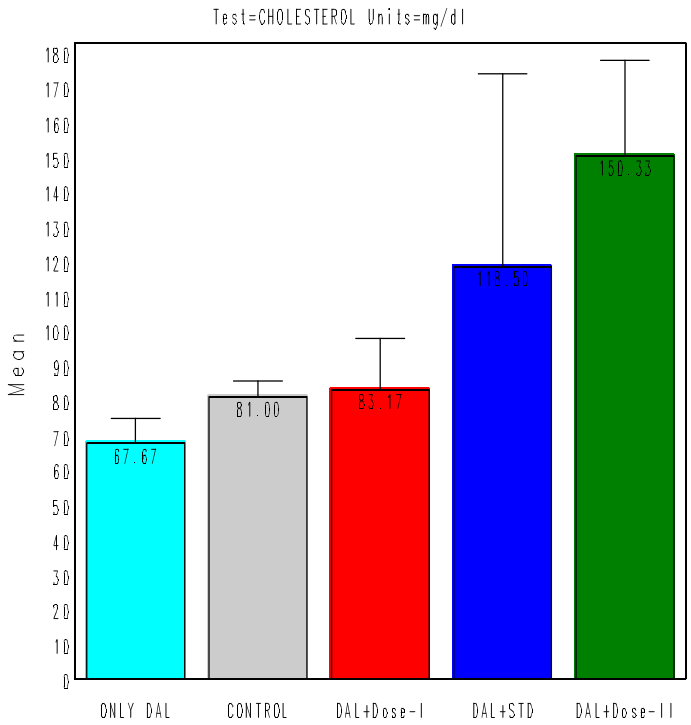
4.11. SERUM IN CHOLESTEROL:

Table 4.15: Shows Serum in CHOLESTEROL levels

Test	Units	CONTROL	DAL+DOSE -I	DAL + DOSE-II	DAL + STD	ONLY DAL
Cholesterol	mgdl	81.00±4.43	83.17±13.96	150.33±26.82a	118.50±52.8a	67.67±6.861

Values are expressed as the mean ± S.E.M., (n = 6) on day 14 of the experiment; [a]*P*<0.05, [b]P<0.01, and [c]P<0.001 between normal and tumor control [d]*P*<0.05, [e]P<0.01, and [f]P<0.001 between tumor control and treated groups and ns- non significant.

CHOLESTEROL



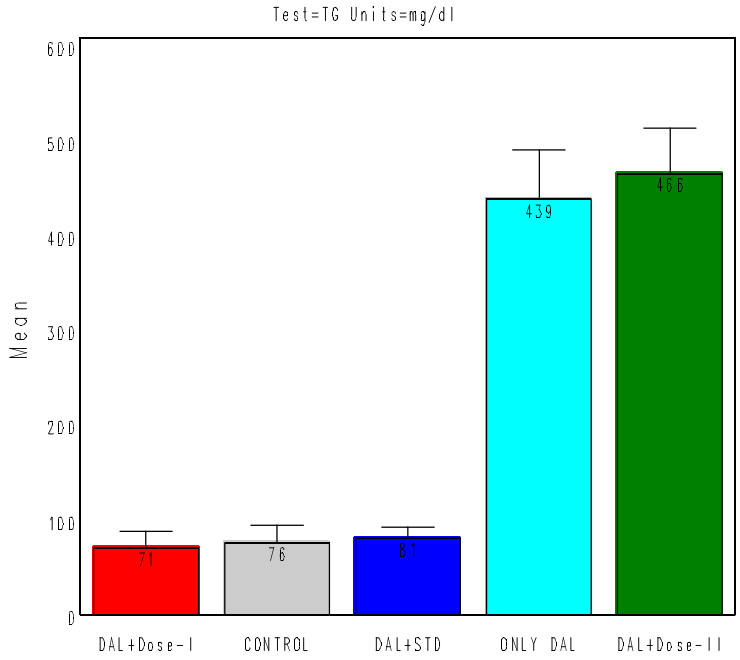
4.12. SERUM IN TRIGLYCERIDES (TGL):

Table 4.16 Shows Serum in TGL levels

Test	Units	CONTROL	DAL+DOSE-I	DAL+DOSE-II	DAL + STD	ONLY DAL
TGL	mgdl	76.15±17.55a	71.22±16.75a	466.00±45.374	80.97±11.20a	438.83±49.44

Values are expressed as the mean ± S.E.M., (n = 6) on day 14 of the experiment; [a]*P*<0.05, [b]P<0.01, and [c]P<0.001 between normal and tumor control [d]*P*<0.05, [e]P<0.01, and [f]P<0.001 between tumor control and treated groups and ns- non significant.

TRIGLYCERIDES (TGL):



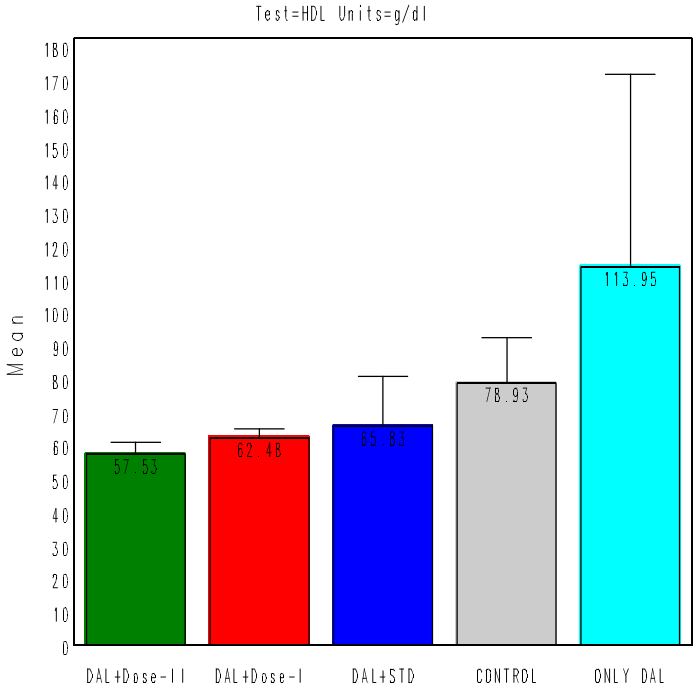
4.13. SERUM IN HDL:

Table 4.17: Shows Serum in HDL levels

Test	Units	CONTROL	DAL+DOSE-I	DAL+DOSE-II	DAL + STD	ONLY DAL
HDL	gdl	78.93±12.93	62.48±2.47a	57.53±3.449a	65.8±14.41a	113.95±55.18

Values are expressed as the mean ± S.E.M., (n = 6) on day 14 of the experiment; [a]*P*<0.05, [b]P<0.01, and [c]P<0.001 between normal and tumor control [d]*P*<0.05, [e]P<0.01, and [f]P<0.001 between tumor control and treated groups and ns- non significant.

SERUM IN HDL



4.14. EFFECT OF METHANOLIC EXTRACT OF LEAVES *OF CASSIA FISTULA LINN* ON IN VIVO ANTIOXIDANT STUDIES IN MICE CHALLENGED WITH DALTONS LYMPHOMA ASCETIC CELLS. ENZYMATIC ANTI – OXIDANT ACTIVITY

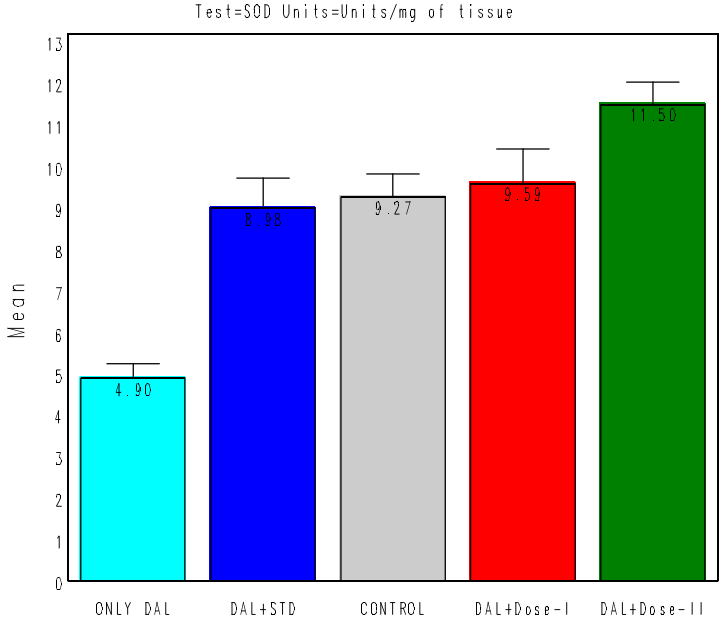
4.14.1. ESTIMATION OF SUPEROXIDE DISMUTASE (SOD) ACTIVITY:

Table 4.18: Shows SOD level.

Test	Units	CONTROL	DAL +DOSE-I	DAL + DOSE-II	DAL + STD	ONLY DAL
SOD	Units/mg of tissue	9.27±0.520 a	9.59±0.795 a	11.50±0.508 a	8.98±0.701 a	4.90±0.326

Values are expressed as the mean ± S.E.M., (n = 6) on day 14 of the experiment; [a]*P*<0.05, [b]P<0.01, and [c]P<0.001 between normal and tumor control [d]*P*<0.05, [e]P<0.01, and [f]P<0.001 between tumor control and treated groups and ns- non significant.

EFFECT OF LEAVES OF *CASSIA FISTULA LINN* ON SOD



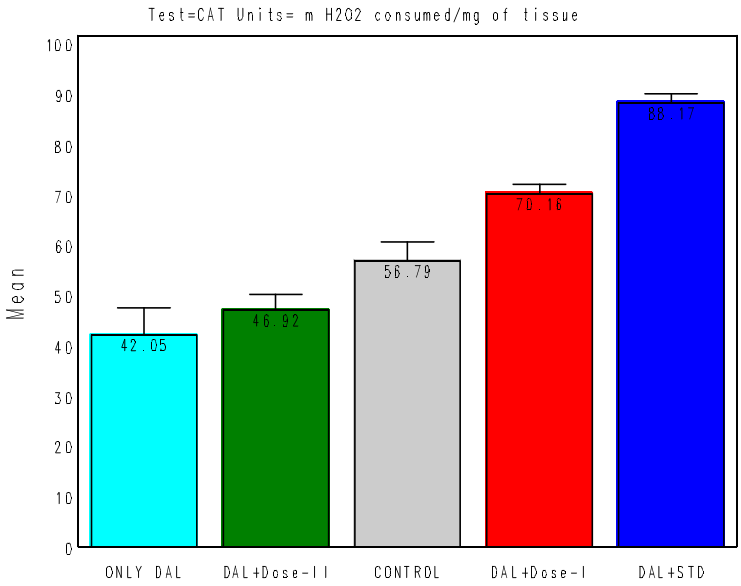
4.14.2. ESTIMATION OF CATALASE ACTIVITY:

Table 4.19: Shows Catalase level.

Test	Units	CONTROL	DAL +DOSE-I	DAL + DOSE-II	DAL + STD	ONLY DAL
CAT	µm H2O2 consumedg of tissue	56.79±3.510a	70.16±1.738a	46.92±3.039	88.17±1.795a	42.05±5.116

Values are expressed as the mean ± S.E.M., (n = 6) on day 14 of the experiment; [a]*P*<0.05, [b]P<0.01, and [c]P<0.001 between normal and tumor control [d]*P*<0.05, [e]P<0.01, and [f]P<0.001 between tumor control and treated groups and ns- non significant.

EFFECT OF LEAVES OF *CASSIA FISTULA LINN* ON CATALASE



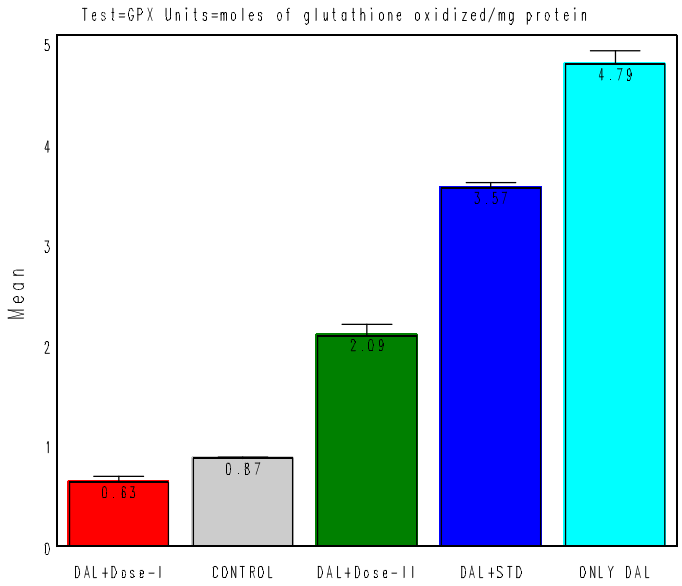
4.14.3. ESTIMATION OF GLUTATHIONE PEROXIDASE (GPX) ACTIVITY :

Table 4.20.Shows GPX level of serum.

Test	Units	CONTROL	DAL +DOSE-I	DAL + DOSE-II	DAL + STD	ONLY DAL
GPX	Moles of glutathione oxidizedg protein	0.87±0.011a	0.63±0.054a	2.09±0.110a	3.57±0.044a	4.79±0.131

Values are expressed as the mean ± S.E.M., (n = 6) on day 14 of the experiment; [a]*P*<0.05, [b]P<0.01, and [c]P<0.001 between normal and tumor control [d]*P*<0.05, [e]P<0.01, and [f]P<0.001 between tumor control and treated groups and ns- non significant.

GLUTATHIONE PEROXIDASE (GPX)]



NON-ENZYMATIC ANTIOXIDANT ACTIVITY

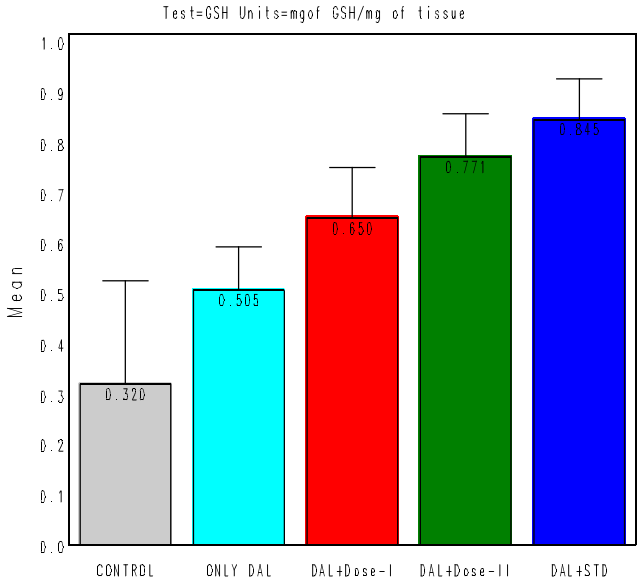
4.14.4. ESTIMATION OF REDUCED GLUTATHIONE (GSH) ACTIVITY:

Table4.21 Shows GSH level of serum.

Test	Units	CONTROL	DAL +DOSE-I	DAL + DOSE-II	DAL + STD	ONLY DAL
GSH	mg of GSHg of tissue	0.32±0.195a	0.65±0.095	0.77±0.082a	0.85±0.077a	0.51±0.082

Values are expressed as the mean ± S.E.M., (n = 6) on day 14 of the experiment; [a]*P*<0.05, [b]P<0.01, and [c]P<0.001 between normal and tumor control [d]*P*<0.05, [e]P<0.01, and [f]P<0.001 between tumor control and treated groups and ns- non significant.

REDUCED GLUTATHIONE (GSH)



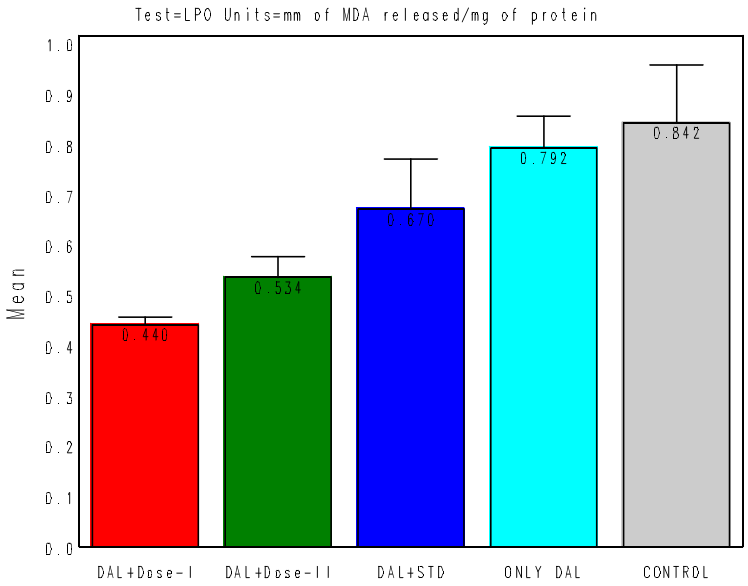
4.14.5. ESTIMATION OF LIPID PEROXIDATION (LPO) ACTIVITY:

Table4.22 Shows GSH level of serum.

Test	Units	CONTROL	DAL +DOSE-I	DAL + DOSE-II	DAL + STD	ONLY DAL
LPO	mm of MDA releasedg of protein	0.84±0.109	0.44±0.015a	0.53±0.040a	0.67±0.095a	0.79±0.060

Values are expressed as the mean ± S.E.M., (n = 6) on day 14 of the experiment; [a]*P*<0.05, [b]P<0.01, and [c]P<0.001 between normal and tumor control [d]*P*<0.05, [e]P<0.01, and [f]P<0.001 between tumor control and treated groups and ns- non significant.

LIPID PEROXIDATION (LPO)



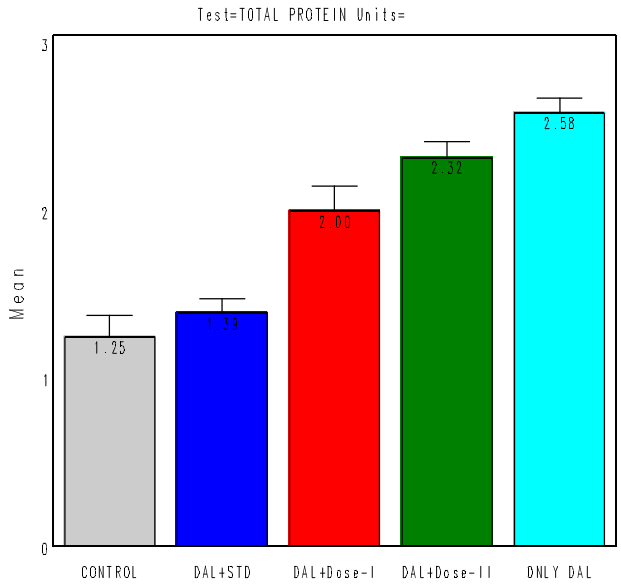
4.14.6. ESTIMATION OF TOTAL PROTEIN:

Table4.23 Shows PROTEIN level in serum.

Test	Units	CONTROL	DAL +DOSE-I	DAL + DOSE-II	DAL + STD	ONLY DAL
TOTAL PROTEIN		1.25±0.123a	2.00±0.143a	2.32±0.092a	1.39±0.081a	2.58±0.087

Values are expressed as the mean ± S.E.M., (n = 6) on day 14 of the experiment; [a]*P*<0.05, [b]P<0.01, and [c]P<0.001 between normal and tumor control [d]*P*<0.05, [e]P<0.01, and [f]P<0.001 between tumor control and treated groups and ns- non significant.

TOTAL PROTEIN



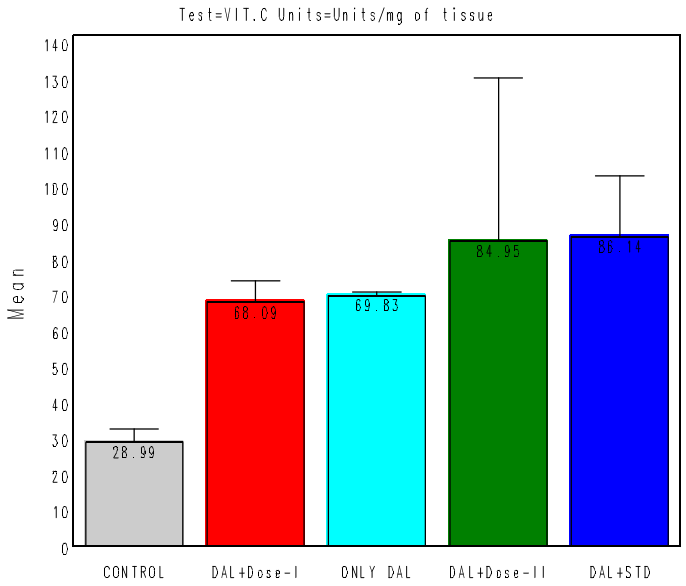
4.14.7. ESTIMATION OF ASCORBIC ACID (VIT.C):

Table4.24 Shows VIT.C level in serum.

Test	Units	CONTROL	DAL +DOSE-I	DAL + DOSE-II	DAL + STD	ONLY DAL
VIT.C	Units g of tissue	28.99±3.448a	68.09±5.486	84.95±43.337	86.14±16.187	69.83±0.915

Values are expressed as the mean ± S.E.M., (n = 6) on day 14 of the experiment; [a]*P*<0.05, [b]P<0.01, and [c]P<0.001 between normal and tumor control [d]*P*<0.05, [e]P<0.01, and [f]P<0.001 between tumor control and treated groups and ns- non significant.

ASCORBIC ACID



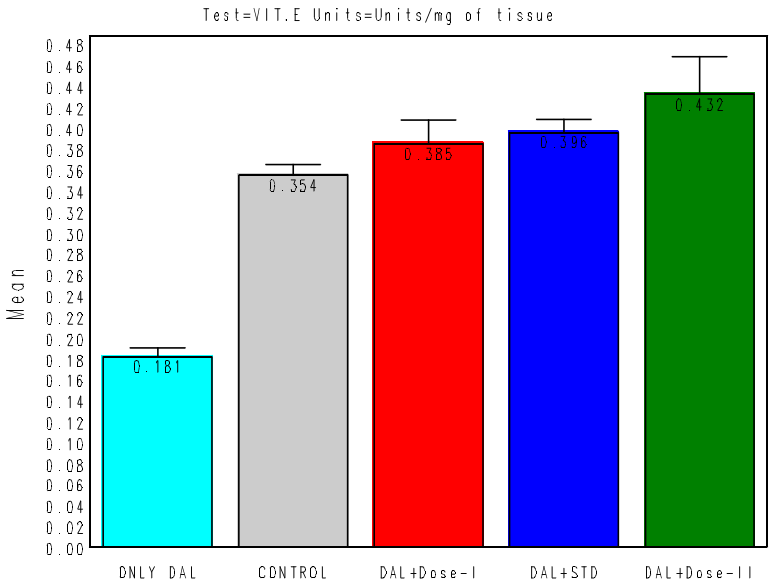
4.14.8. ESTIMATION OF VIT.E (α-TOCHOPHREOL):

Table 4.25 Shows VIT.E level in Serum.

Test	Units	CONTROL	DAL +DOSE-I	DAL + DOSE-II	DAL + STD	ONLY DAL
VIT.E	Units g of tissue	0.35±0.010a	0.38±0.021a	0.43±0.034a	0.40±0.012a	0.18±0.009

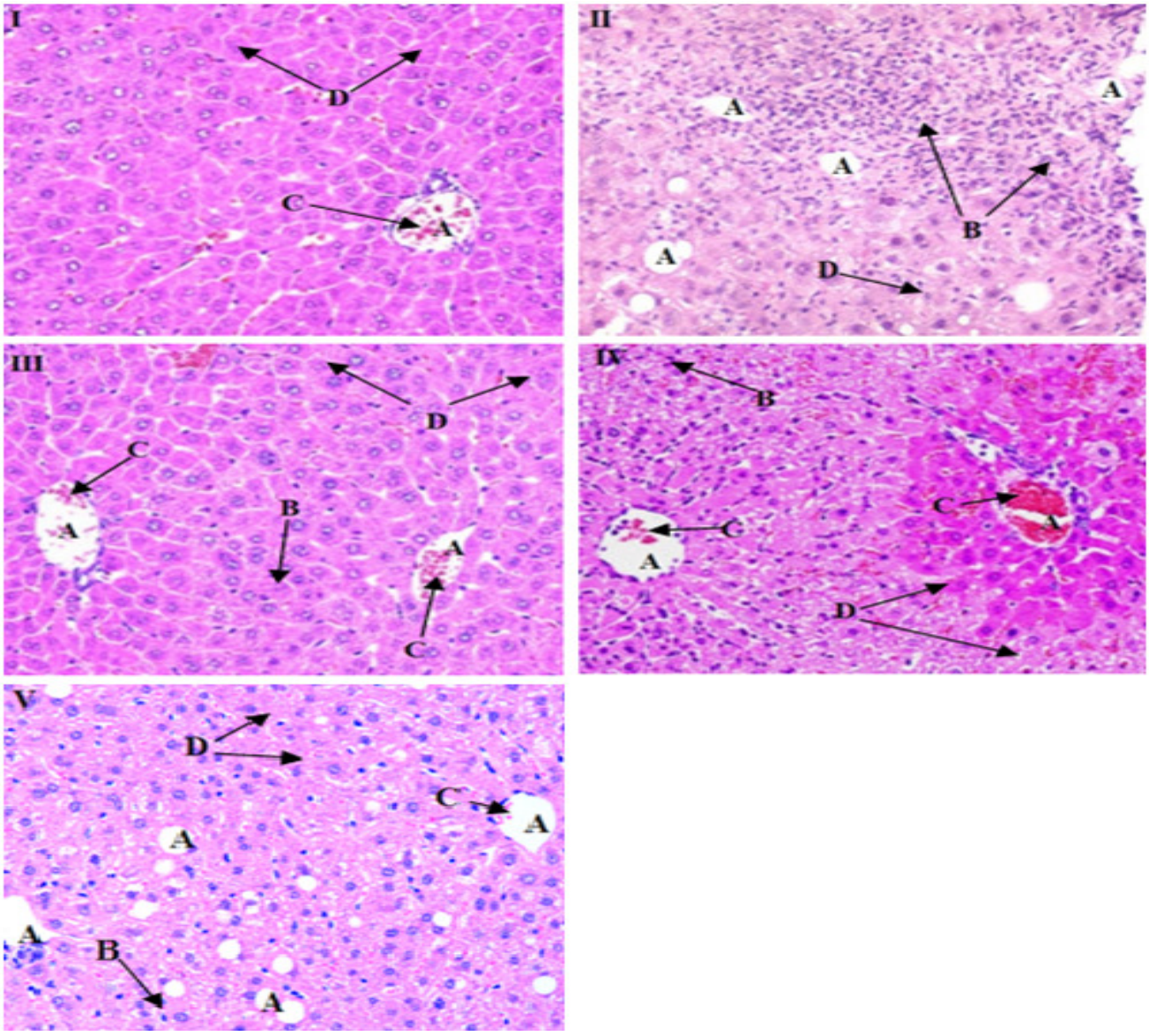
Values are expressed as the mean ± S.E.M., (n = 6) on day 14 of the experiment; [a]*P*<0.05, [b]P<0.01, and [c]P<0.001 between normal and tumor control [d]*P*<0.05, [e]P<0.01, and [f]P<0.001 between tumor control and treated groups and ns- non significant.

α-TOCHOPHREOL



EFFECT OF METHANOLIC EXTRACT OF LEAVES *OF CASSIA FISTULA LINN* ON HISTOPATHOLOGICAL ANALYSIS IN MICE CHALLENGED WITH DALTONS LYMPHOMA ASCETIC CELLS.

Figure 4.3: Histology of liver stained with Haematoxylin-Eosin (×100) of mice from the different groups, on day 14 of the experiment (n = 6): I: Normal; II: DAL control; III: DAL + 5-FU,20 mg/kg body weight; IV: DAL + CF250 mg/kg bodyweight; e: DAL +



5. DISCUSSION

The preliminary phytochemical studies of leaves of *Cassia fistula linn* indicated the presence of several triterpenoids, phenols, flavonoids, tannins, glycosides, and so on. The

observed antitumor, hepatoprotective, and antioxidant activities may be due to the presence of any of these compounds in leaves of *Cassia fistula linn.*

Bioactive antioxidant levels:

Flavonoids are a group of effective antioxidants which are present abundantly throughout the plant kingdom. Flavonoid and related compound are effective in scavenging DPPH radical, hydroxyl radical and in metal-chelating capacity Flavonoids are found to exhibit numerous biological activities like vasodilatory, anticarcinogenic, antiinflammatory, antibacterial, immune-stimulating, antiallergic, and antiviral effects (Middleton &Kandaswami, 1992)[14,15].

Tannins, the high molecular weight phenols, act as a good scavenger of free radical either by donating hydrogen atom or by reducing them. This property is attributed by the molecular weight, the number of aromatic rings and nature of hydroxyl group's substitution and specific functional groups present in the tannins. The results show that the methanol extract contains more tannin.[55]

Phenols are very important plant constituents because of their radical scavenging ability due to the hydroxyl groups. **Polyphenols** are known to exhibit a variety of biological actions such as free radicals scavenging, metal chelation, modulation of enzyme activity and more recently to effect signal transduction, activation of transcription factors and gene expression.[56]

Free radicals may be defined as any species that is capable of independent existence and possessing one or more unpaired electrons. These radicals can react with other molecules in many ways. The net effect is that radical donates its unpaired electron to another molecule and this molecule then becomes radical.

The **DPPH** assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm (purple colour). As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, e.g., a free radical-scavenging antioxidant, the absorption strength is

decreased and results in decolorization (yellow colour) with respect to the number of electrons captured[57]More the decolorization more is the reducing ability. This test has been the most accepted model for evaluating the free radical scavenging activity of any new drug. The percent DPPH scavenging activities of leaves of cassia fistula linn extracts was summarised in **(PAGE.)**. The scavenging activity **(EC_{50})** was found to be **44.52µg/ml**

ABTS The decolorization of $ABTS^{\bullet+}$cation radical is an unambiguous way to measure the antioxidant activity of phenolic compounds. Thus the ability of a compound to scavenge $ABTS^{\bullet+}$ radical can demonstrate oxygen radical absorbance capacity. Methanol extracts of A. pavonina showed very potent ABTS radical scavenging activity **(EC_{50})** was found to be **17.46 µg/ml**.

FRAP assay measures the reducing ability of antioxidants against oxidative effects of reactive oxygen species. Electron donating anti-oxidants can be described as reductants and inactivation of oxidants by reductants can be described as redox reactions. Total antioxidant power may be referred analogously to total reducing power. In the current study methanol extracts of A. pavonina exhibited about 7.17 µg of standard is equivalent to 50 µg of leaves of cassia fistula linn extract (143.4 mg/g of extract).

TRAP, Methanol extracts of A. pavonina showed very potent radical scavenging activity **(EC_{50})** was found to be **62.95µg/ml.**

Methanolic extract of Leaves of *Cassia fistula linn* was traditionally used in the treatment of tumors. The present investigation was carried out to evaluate the antitumor activity of the leaves of *Cassia fistula linn* in DAL tumor bearing mice. The reliable criteria for judging the value of an anticancer drug is the prolongation of the life span of animals. In DAL-tumor-bearing mice, a regular rapid increase in ascitic tumor volume was observed[62]. The DAL-bearing mice orally administered leaves of *Cassia fistula linn* at 250 and 500 mg/kg body weight showed significant change in the average

life span compared to animals of the tumor control group. However, the percentage increase in body weight, tumor cell volume, and number of viable tumor cells were found to be significantly less than the tumor control animals, indicating the anticancer nature of the extract. These results could indicate either a direct cytotoxic effect of leaves of *Cassia fistula linn* on tumor cells as evidenced by the in vitro studies or an indirect local effect, which may involve macrophage activation and vascular permeability inhibition. Hence, the observed antitumor nature of leaves of C*assia fistula linn* may be due to the cytotoxic properties.

To investigate if the inhibitory effect of leaves of *Cassia fistula linn* on DAL tumor was local or systemic, the effect of i.p. injection of leaves of *Cassia fistula linn* in another experimental system, DAL-induced solid tumor, was tested. The solid tumor was inhibited by treatment with leaves of *Cassia fistula linn*, suggesting that the inhibitory effect is related not only to a local cytotoxic effect but also with the systemic effect of leaves of *Cassia fistula linn*.

The reversal of Hgb content, RBC, Total WBC, and differential count of WBC by the leaves of *Cassia fistula linn* treatment towards the values of the normal group clearly indicates that leaves of *Cassia fistula linn* possessed protective action on the haemopoietic system. However, the elevation of WBC levels may be due to its adverse effect on the haemopoietic system[58].

It was reported that the presence of tumors in the human body or in experimental animals is known to affect many functions of the liver and kidney. The significantly elevated levels of ALP, TB, and decreases in the levels of SOD in serum, liver, or kidney of tumor-inoculated animals indicated liver damage and loss of functional integrity of cell membranes[59] . This was also indicated by the significant increase in the levels of ASAT, ALAT, CR, and albumin and decreases in TGL, TP, TC, and CAT in the liver and kidney tissues of the tumor control group. The significant reversal of these changes towards the normal by leaves of cassia fistula linn treatment in most of the cases demonstrated the potent hepato protective and antioxidant nature of leaves of *Cassia fistula linn.*

The antioxidant nature of leaves of *Cassia fistula linn* was also evident by the in vitro studies. Plants with high total phenol content are known to possess strong

antioxidant properties[60]. The observed antioxidant activity may be due to the high phenolic content of the extract. Hepato cellular necrosis leads to high levels of ASAT and ALAT, which are released from liver into the blood. ALP activity, on the other hand, is related to the functioning of hepatocytes. Increase in its activity is due to increased synthesis in the presence of increased biliary pressure[61]. Reduction in the levels of these towards the respective normal values in liver and kidney tissues is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by tumor inoculation.

Lipid peroxidation refers to the oxidative degradation of lipids. It is the process whereby free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. It most often affects polyunsaturated fatty acids, because they contain multiple double bonds in between which lies methylene -CH2- groups that possess especially reactive hydrogen's.

Liver damage leads to the accumulation of fat and necrosis in the centrilobular region of the liver. As a consequence, the microsomal enzyme activities are found to decrease and due to lipid peroxidation, the water soluble enzymes leak into plasma from the liver. It is shown by the significant decrease in triglycerides and proteins in the liver and kidney of the tumorinoculated animals[62]. These changed parameters were restored towards the normal levels by leaves of cassia fistula linn treatment, indicating its hepatoprotective nature. The decreased bilirubin level brought about by the leaves of *Cassia fistula linn* treatment indicates the normal functional conditions of the liver.

It was observed that tumor cells produced more peroxides when they proliferate actively after inoculation of tumor. This rise in peroxides indicated the occurrence of intensification of oxygen free radical production[62]. Cells which are equipped with enzymatic antioxidant mechanisms play an important role in the elimination of free radicals. High levels (up to 0.05 μmol/h per 104 cells) of H2O2 are constitutively released from a wide variety of human tumors [64]. SOD and CAT are involved in the clearance of superoxide and H_2O_2.

Decrease in SOD and CAT activities described in tumors is regarded as markers of malignant transformation. Lowered activities of SOD and CAT were reported in several cancers[65]. The significant elevation of SOD and CAT by the extract treatment confirms the potent antioxidant activity of the leaves of *Cassia fistula linn* The elevation of lipid peroxidation is known to be associated with cancer. MDA, the end product of lipid peroxidation, was reported to be higher in cancer tissue than in normal tissues [66]. TBARS levels in the blood and the tested tissues in tumor control animals were found to be higher than those in normal animals. The leaves of *Cassia fistula linn* treatment caused a significant reduction in TBARS levels in all the samples tested. This indicates the reduction in free radical yield and subsequent decrease in harm and damage to the cell membrane and decrease in MDA production.

The histological examination of the liver and kidney of DAL-inoculated animals showed marked changes indicating the toxic effects of this tumor. Leaves of *Cassia fistula linn* may cause firstly an antitumor effect and then influence biochemical parameters. Plant-derived extracts containing antioxidant principles with cytotoxicity towards tumor cells and antitumor activity in experimental animals were reported [67]. Plants with potent antitumor, hepatoprotective, and antioxidant properties were also reported [68].

6. CONCLUSION

The present investigation was carried out in methanolic extracts of leaves of *Cassia fistula linn* increased the life span of Dal tumor bearing mice and improve

hematological and antioxidant status and decreased the lipid peroxidation and thereby enhance the endogenous antioxidant enzymes in the liver. The biochemical and histological studies supported its anti tumor and antioxidant properties of methanolic extracts of leaves of *Cassia fistula linn.*

Further studies to characterize the active principle and elucidate the mechanism of action of methanolic extracts of leaves of *Cassia fistula linn.*

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